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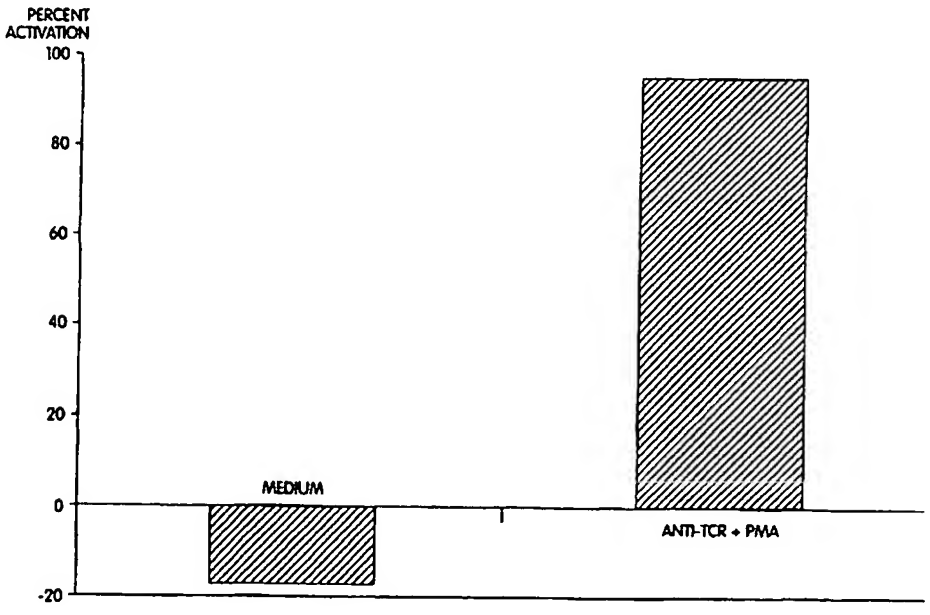

(57) Abstract

This invention provides a multi-step, high throughput primary screen to identify immune regulators of T cell activation for use as therapeutic agents. Compositions or compounds are screened for their ability to stimulate or inhibit the expression of a reporter gene operatively linked to specific transcriptional control sequences in T cells. A composition or compound identified as an immune stimulator or inhibitor by the primary screen of this invention can then be further characterized to determine the target molecule on which the composition or compound acts to regulate T cell activation and T cell activation-dependent processes such as apoptosis.

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METHOD OF ISOLATING REGULATORS OF T CELL ACTIVATION

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part application of United States Serial No. 08/639,286, filed April 25, 1996.

GENERAL FIELD OF THE INVENTION

This invention relates generally to methods for identifying immunosuppressive and immunoactivating compounds. In particular, described herein is a multi-step, high throughput screening assay which provides a means to identify and isolate from mixtures or libraries of compounds of known or unknown structure and activity individual compounds that are capable of inhibiting or stimulating transcription of genes regulated by the activation of T cells which occurs upon binding or cross-linking of the T cell antigen receptor expressed on the surface of T cells.

BACKGROUND OF THE INVENTION

Eliciting an immune response that is highly specific for a particular antigen usually requires the participation of certain T cells (i.e., helper T cells) to stimulate a response by the various cells of the immune system. One of the first steps in this process is for T cells themselves to be activated to proliferate and to produce cytokines which exert their influence on the immune system generally. Such T cell activation normally occurs when a T cell antigen receptor (TCR) on the surface of a T cell recognizes and binds to a particular peptide epitope of a foreign protein antigen which is associated with a class II major histocompatibility complex (MHC) on the surface of an antigen-presenting cell. The binding of a TCR to a cognate peptide associated with an MHC on an antigen-presenting cell triggers a signaling cascade that stimulates transcription of cytokines, such as interleukin-2 (IL-2), that are essential for early activation of T cells as well as the subsequent activation of a variety of other cells of the immune system including B cells, macrophages, and eosinophils.

The successful transduction of the activation signal from the binding of the TCR on the surface of a T cell to the nucleus where cytokine gene transcription occurs involves a complex network of intracellular reactions (see, for example, Figures 1 and 2). Early activation cytokines, such as IL-2, which are released from the T cell, activate the T cell itself to proliferate (autocrine proliferation) and also activate other cells of the immune system,

such as B cells, macrophages, eosinophils. The expression of these early activation cytokines is regulated by several transcription control factors that form a number of complexes which bind at specific (enhancer) transcription control sequences associated with each cytokine gene (Schwartz, *Cell, 7*: 1065 - 1068 (1992)). Thus, the intracellular transduction of a signal for T cell activation leads to the activation of transcription factors which bind at specific transcription control sequences to stimulate cytokine gene expression in the T cell.

The Nuclear Factor of Activated T Cells (NF-AT) (see, for example, Shaw et al., *Science, 241*: 202 - 205 (1988); Rao, *Immunol. Today, 15*: 274 - 281 (1994)) is a transcription factor that is critical to the transcription of early activation cytokines such as interleukin-2 (see, for example, Durand et al., *Mol. Cell. Biol., 8*: 1715 - 1724 (1988); Shaw et al., *Science, 241*: 202 - 205 (1988); Hoey et al., *Immunity, 2*: 461 - 472 (1995) (IL-2); Cockerill et al., *Proc. Natl. Acad. Sci. USA, 90*: 2466 - 2470 (1993) (IL-3); Chuvpilo et al., *Nucl. Acids Res., 21*: 5694 - 5704 (1993); and Szabo et al., *Mol. Cell. Biol., 13*: 4793 - 4805 (1993) (IL-4)). During the transduction of the activation signal from the bound TCR on the T cell surface, calcineurin, a calmodulin-dependent phosphatase, dephosphorylates cytoplasmic NF-AT which then can pass into the nucleus where it associates with at least one other factor (such as, AP-1 or CREB) to form the NF-AT transcription control complex. The NF-AT transcription control factor then can bind to the NF-AT transcription control sequence in the IL-2 enhancer region to activate transcription of the IL-2 gene. Other transcription factors involved in the stimulation of transcription of the IL-2 gene include fos and jun proteins that form an AP-1 transcription factor complex, NF-κB, and an octamer-binding complex transcription factor (Oct) (Schwartz, *Cell, 71*: 1065 - 1068 (1992)). Such transcription control factors do not act independently of one another as evidenced by the fact that AP-1 must bind in the IL-2 enhancer region in order for NF-AT to bind at its cognate sequence. The exact composition and binding associations of the various transcription factors involved in cytokine expression during T cell activation remain to be fully elucidated (see, for example, Schwartz, *Cell, 71*: 1065 - 1068 (1992); Rao, 1994). Clearly, there are numerous potential target sites between the TCR and the transcription of cytokine genes at which a compound could act to modulate (that is, either to stimulate or to inhibit) the T cell activation process (see, for example, Figures 1 and 2).

Therapeutic regulation of the immune system can be divided into two major clinical categories. The first is immune suppression (or inhibition), which is useful, for example, in

the prevention of organ and tissue transplant rejection, allergies, and autoimmune diseases. Current therapies for transplant rejection employ either cyclosporin A (CSA) or FK-506, in combination with steroids and azathioprine (so-called "triple therapy"). A target at which cyclosporin A and FK-506 act is the Ca^{++}-dependent phosphatase calcineurin (see, Liu et al., *Immunol. Today, 291*: 290 - 295 (1993); Figures 1 and 2). However, these compounds have an important drawback in that they are generally toxic to all cells, possibly due to inhibition of expression of genes that are necessary for vital functions required by many cells. In addition, owing to the critical roles in detoxification and filtering of the blood, liver and kidney cells are especially susceptible to the toxic effects of such compounds in the circulatory system. Accordingly, transplant patients are often taken off currently available immunosuppressive therapy to prevent renal or liver failure and death resulting from such cell toxicity, which often leads to loss (rejection) of the graft.

The second clinical category of immune system regulation is immune activation (or stimulation), which is useful, for example, to assist in tumor rejection or to boost immune responses to infectious agents such as bacteria, parasites, and viruses, in particular human immunodeficiency virus (HIV), which causes acquired immune deficiency syndrome (AIDS). No such T cell activator compounds are in current clinical use.

There is a need, therefore, for compounds that act as immune regulators specific for T cell activation without an attendant general cellular toxicity.

SUMMARY OF THE INVENTION

A multi-step, primary screen is described that provides the means and methods for identifying and isolating compositions or compounds that act as immune regulators to inhibit or stimulate T cell activation. The primary screen of this invention can be used to screen any composition or compound that can be added to and dissolved or dispersed in cell cultures. Thus, the compositions and compounds screened by the primary screen of this invention may range from relatively crude preparations such as extracts of microbiological cultures, which may contain multiple unknown components, to relatively pure substances such as synthetically produced organic compounds of known chemical structure.

The individual steps of the primary screen utilize T cells transfected with recombinant plasmid constructs containing a reporter gene, such as the luciferase gene (LUC), encoding an easily detected protein and which is operatively linked to, i.e., under the transcriptional

control of, either specific transcription control sequences from cytokine genes, such as the interleukin-2 (IL-2) gene, which are normally involved in the early activation of T cells, a transcription control sequence of a ubiquitously expressed gene which is important for cell viability, or a sequence which is specifically bound by a transcription factor involved in the regulation of one or more genes involved in early activation of T cells. In a preferred embodiment, the initial step (Activation Assay) determines whether a composition or compound can inhibit or stimulate T cell activation by screening for the ability to inhibit or stimulate expression of a reporter gene operatively linked to the promoter/enhancer region of a gene involved in early activation of T cells. Then, a second assay step (Toxicity Assay) is used to determine whether the composition or compound is likely to have an activity that is generally toxic to all or many cells. In the Toxicity Assay of the primary screen of this invention, the transcription of a reporter gene is under the control of a promoter sequence from a gene, such as β-actin, which is constitutively expressed in all or most cells of the human body and which is important for cell viability. Compositions or compounds that inhibit or stimulate expression of the reporter gene construct in the Toxicity Assay are discarded as having a general toxicity, except for anti-apoptotic compounds which stimulate reporter gene expression in activated T cells when the Toxicity Assay is run for greater than 8 hours. In particular, such compositions or compounds can be identified as anti-apoptotic agents if they stimulate reporter gene expression in activated but not in unactivated T cells used in the Toxicity Assay. In a third step (Specificity Assay) of the preferred embodiment, an assay is employed that indicates whether the composition or compound is capable of affecting a particular transcription factor's control over cytokine gene expression.

In a more preferred embodiment, each step of the primary screen of this invention uses Jurkat cells transfected with an appropriate recombinant plasmid containing the luciferase reporter gene operatively linked to either an IL-2 promoter/enhancer sequence (IL-2LUC) for the Activation Assay; a β-actin promoter region (β-actinLUC) for the Toxicity Assay; or a plurality of tandem NFAT, AP-1, or NFκB transcription factor binding sequences from the IL-2 promoter region (NFATLUC, AP-1LUC, NFκBLUC) for the Specificity Assay.

According to the invention, a composition or compound that affects (stimulates or inhibits) reporter gene expression in the assays of the first (Activation Assay) and third (Specificity Assay) steps of the primary screen and which also exhibits no significant effect

on reporter gene expression in the assay of the second step (Toxicity Assay) when run for less than 8 hours is considered an immune regulator based on the primary screen described herein. Subsequent secondary assays are provided to further characterize the mode of action of the immune regulator as well as the likely identity of the molecular target(s) acted on by the immune regulator.

Another advantageous aspect of the invention is that the cell-based format of each assay step of the primary screen requires that a compound, a composition, or a component of the composition be capable of binding to or passing into the cell. Accordingly, the primary screen described herein provides an especially efficient means of screening for compositions and compounds that have immune regulatory activity by affecting a target molecule that plays a role at some point in the pathway for transducing the signal for T cell activation from TCR or the associated CD3 molecule on the surface of T cells to genes involved in early T cell activation in the nucleus of the cells. Furthermore, each cell-based assay is carried out under conditions of T cell activation initiated by binding or cross-linking the TCR (or the associated CD3) on the surface of the T cell, thereby providing a condition resembling the physiological conditions of normal T cell activation. Each step of the primary screen also utilizes a reporter gene the expression of which is easily detected and quantified, such as the luciferase gene, thereby providing a high degree of selectivity and sensitivity for detecting a composition or compound which has either an immune stimulatory or inhibitory activity.

The efficiency, selectivity, and sensitivity of the primary screen described herein has been demonstrated by screening libraries, some containing thousands of synthetic compounds, and identifying candidate immune regulators from such libraries that act at target molecules in the T cell activation signaling cascade. Advantageously, the primary screen of this invention permits the identification of immune regulators that act at any of a variety of possible sites (target molecules) along the signal transduction pathway for T cell activation, including those target molecules that are distinct from the site of action of the well known immune inhibitors cyclosporin A and FK506. The primary screen of the invention has also been used to screen libraries consisting of thousands of compositions of fungal culture extracts containing unknown components, leading to the identification of compositions of fungal culture extracts that exhibit immune regulatory activity. The primary screen can also be used to identify active fractions during purification of compounds from mixtures or compositions.

This invention also provides a cell-based assay for identifying immune regulators which are inhibitors of apoptosis (anti-apoptotic agents). Each step of this assay for inhibitors of apoptosis involves incubation of T cells with a composition or compound for greater than 8 hours. The first step of the assay determines whether a composition or compound stimulates expression of a reporter gene in an activated T cell transfected with a reporter gene construct in which a promoter/enhancer region of a cytokine gene involved in early T cell activation, such as IL-2, is operatively linked to the structural coding sequence of the reporter gene. A composition or compound that stimulates reporter gene expression in the first step is then assayed in the second step to determine whether the composition or compound stimulates expression of a reporter gene in both activated and unactivated T cells transfected with a reporter gene construct, wherein the transcriptional control sequence of a ubiquitously expressed gene, such β-actin, is operatively linked to the structural coding sequence of the reporter gene. A composition or compound that stimulates reporter gene expression in the activated T cells in the first step, in the activated T cells of the second step, but not in the unactivated T cells of the second step is selected as an immune regulator which is an anti-apoptotic agent. In a preferred embodiment of this assay, the reporter gene is the luciferase gene.

Immune regulators identified by the methods of this invention are potential therapeutic agents for regulating T cell activation and the various clinically significant phenomena dependent on T cell activation.

These and other features and advantages of the present invention may be better understood by considering the following detailed description of the invention, including certain preferred embodiments and examples thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts a general scheme for certain portions of the signaling cascade involved in the intracellular transduction of a T cell activation signal initiated by the interaction of a T cell antigen receptor (TCR) on the surface of a T cell to a peptide antigen in association with a class II major histocompatibility complex (MHC) on an antigen presenting cell leading to the expression of IL-2, a cytokine involved in early T cell activation. Arrows depict transduction of a signal for T cell activation from one molecule to another or to another pathway. The "+" sign indicates an activating event. The "-" sign indicates an inhibiting

event. A "?" indicates that further signal transduction pathways, effectors, or the mode of action in the T cell are currently not known. The protein pp120 (phosphoprotein 120) and cdc2 were identified during the secondary assays as targets for cyclosporin A and FK506. Numbers indicate sites in the signaling cascade for which a secondary assay is suggested for further characterization of compositions or compounds identified as immune regulators in the primary screen.

Figure 2 depicts another scheme of the intracellular transduction of the signal for T cell activation illustrating the envisioned transcriptional control mechanisms of the IL-2, jun, c-fos, and Fra genes. Solid arrows depict various events in activation (for example, DG/Ca^{+2} → PKC), translocation (e.g., NFAT-PPP → NFAT; ERK → ERK-PP), phosphorylation (for example, FRK → PP-fos), dephosphorylation (for example, JunPase - jun-PP), and gene transcription (see hooked arrows, IL-2, jun, c-fos, Fra genes). Thick dashed arrow shows point of presumptive interaction ("crosstalk") between Ras and JNK pathways. Thin dashed arrows show activation-dependent events reported from other cell lines. "XTcell" adjacent to calcineurin indicates a T cell specific, but unknown, activation event.

Figure 3 shows a flow chart of a preferred embodiment of the Primary Screen for Immune Regulators according to this invention.

Figure 4 shows a flow chart of suggested secondary screening assays for further characterization of compounds identified in a primary screen for immune regulators.

Figure 5 shows a flow chart for a primary screen for immune stimulators, a screen for anti-apoptotic agents, and suggested secondary screening assays.

Figures 6A and 6B show the structures of eight arylidene diamide (oxazolone) derivative compounds identified as immune inhibitors from a library of approximately 8,000 molecules using the Primary Screen of this invention. The structures depicted in Figures 6A and 6B are intended to cover all isomeric forms implied by the structures as drawn. Compounds in Figures 6A and 6B are designated by "AT" numbers and corresponding "AQ" numbers which can be used to obtain the compounds from a commercial supplier (ArQule, Inc., Medford, MA): AT-1 is AQ32778, AT-2 is AQ32705, AT-3 is AQ32785, AT-4 is AQ32809, AT-5 is AQ32687, AT-6 is AQ29268, AT-7 is AQ25958, and AT-8 is AQ588.

Figure 7 shows the results of an Activation Assay for one plate in the Primary Screen for immune regulator compounds. Results plotted as Light Units (counts per second) from luciferase activity (vertical axis) versus control samples (medium = no stimulation, TCR =

anti-TCR antibody/PMA stimulation) and row letter of assay plate (horizontal axis). Numbers indicate columns on assay plate.

Figure 8 shows the results of a Toxicity Assay for compound AT-7 identified as a putative immune suppressor from the Activation Assay of the Primary Screen. Results are plotted as light units from luciferase activity (vertical axis) versus stimulation conditions (horizontal axis): medium (no stimulation), TCR (16G8 Mab/PMA stimulation), CsA (cyclosporin A).

Figure 9 shows the results of a Specificity Assay for compounds at indicated concentrations in the Primary Screen. Results are plotted as Per Cent Inhibition of luciferase activity (vertical axis) versus compound designation (horizontal axis).

Figure 10 shows the results of a Ca^{++} Ionophore/PMA activation assay for compounds identified in the Primary Screen. Results are plotted as Per Cent Inhibition of luciferase activity (vertical axis) versus compound designation (horizontal axis).

Figure 11 shows the results of a Splenocyte Proliferation Assay for splenocytes from naive mice stimulated in vitro with Concanavilin A (Con A) at indicated concentrations in the presence of compounds identified in Primary Screen. Results are plotted as Per Cent Inhibition of ^{3}H-thymidine incorporation (vertical axis) versus compound designation (horizontal axis).

Figure 12 shows the in vitro proliferation of splenocytes from Sperm Whale Myoglobin (SWM)-primed Balb-c mice injected with indicated amounts of AT-2 compound identified in the Primary Screen or with cyclosporin A. Results are plotted as stimulation Indices (see text) (vertical axis) versus SWM concentration (horizontal axis).

Figure 13 shows a Delayed-Typed Hypersensitivity (DTH) response in Sperm Whale Myoglobin-primed Balb-c mice injected with indicated amounts of compound AT-8 identified in the Primary Screen or cyclosporin A. Results are plotted as edema (mm, vertical axis) versus group (N, horizontal axis). All groups (N) received SWM, except the "no antigen group".

Figure 14 shows results of repeated assays of immune activators identified in the Primary Screen for immune regulator compounds (see Example 2). Results are plotted as Per Cent Activation of luciferase activity (vertical axis) versus compound designation (horizontal axis).

Figure 15 shows results of a Toxicity Assay of immune activators identified in the

Primary Screen for immune regulator compounds (see Example 2). Results are plotted as Per Cent Activation of luciferase activity (vertical axis) versus compound designation (horizontal axis).

Figure 16 shows results of Figure 15 but omitting data of compound designated "O".

Figure 17 shows results of a Specificity Assay of immune activators identified in the Primary Screen for immune regulator compounds. Results are plotted as Per Cent Activation of luciferase activity (vertical axis) versus compound designation (horizontal axis).

Figures 18A - 18F show the results of an Apoptosis Assay using Flow Cytometry to detect fluorescein isothiocyanate (FITC)-labeled Annexin V binding (staining) to exposed phosphatidylserine (PS) on the surface of Jurkat cells. Data in Figures 18A - 18F are plotted as cell counts (Counts) versus fluorescence (FL1-Height). Cells were incubated with no compound (control, Figure 18A); with antibody to human Fas (α-human Fas) to induce apoptosis (positive control, Figure 18B); with test compound M3.5H8 at 60 ng/ml (Figure 18C) or at 2 μg/ml (Figure 18D); or with test compound M4.6H5 at 60 ng/ml (Figure 18E) or 5 μg/ml (Figure 18F). M3.5H8 and M4.6H5 are compounds identified as immune regulators by screening a library of 3,840 organic compounds synthesized by combinatorial chemistry using the primary screen as described in Example 4. M1 indicates the total range of fluorescent signal surveyed as an indication of the total number of cells in the assay. M2 indicates the range of fluorescent signal surveyed to detect apoptotic cells in the assay.

Figure 19 shows production of IL-2 from anti-CD3 treated splenocytes in the presence of immune stimulator composition XG08 identified by a primary screen in Example 7.

Figure 20 shows expression of luciferase (light units) versus stimulation conditions versus concentration of cyclosporin A (CsA, ng/ml) in cells transfected with β-actinLUC and incubated for 18 hours under the indicated conditions.

Figure 21 shows stimulation of expression of luciferase in activated and unactivated Jurkat cells transfected with β-actinLUC. Results are plotted as Percent Activation (vertical axis) versus compound (horizontal axis) under activated and unactivated conditions over an 18 hour incubation period.

DETAILED DESCRIPTION

Described herein is a multi-step, cell-based, high throughput primary screen which permits the identification and isolation of compositions or compounds that stimulate or inhibit T cell activation. As used herein, a "composition" that may be screened using a

primary screen according to this invention is any substance that can be dissolved or dispersed in the cell cultures of each step of the primary screen of this invention. Thus, the term "composition" encompasses a wide range of substances that may be screened according to the protocol of this invention, ranging from relatively crude and complex mixtures, such as crude extracts of microbiological cultures having potentially multiple, undefined components, to pure, synthetically produced, individual organic compounds of known chemical structure, such as members of an array or library of compounds produced by combinatorial chemistry. In the absence of further description, the term "compound" is used to indicate that at least one molecular species, of known or unknown structure or chemical formula, is present in a sample being screened or assayed. Thus, a composition screened according to the methods of this invention may contain one or more compounds. Any assay described herein may be used to screen or further characterize compositions or individual compounds. Compositions or compounds that are identified by the primary screen of this invention as having an inhibitory or stimulatory effect on T cell activation may be used to regulate immune system activity in humans and animals or to promote or suppress T cell activity not only *in vivo*, but also *in vitro* or *ex vivo*. Such compositions or compounds are called immune regulators and, more specifically, are further termed immune stimulators/activators or immune inhibitors/suppressors depending on whether they effectively increase or suppress T cell activation, respectively, as indicated by the primary screen of this invention. The primary screen described herein is designed to detect at least one of these activities of such an immune regulator. Thus, immune regulatory compositions or compounds identified and isolated according to this invention include, but are not limited to, small organic compounds, proteins, polypeptides, and multi-component preparations or extracts from natural sources, such as bacterial or fungal cultures.

The primary screen of this invention also indicates whether a composition or compound is likely to have the undesirable property of being generally toxic to all or most cells, and, thus, should be screened out as not therapeutically useful even though it exhibits an immune stimulatory or inhibitory activity. Furthermore, once a particular composition or compound has been identified and characterized by the primary screen of this invention as a candidate immune regulator, a more detailed characterization of the composition or compound and its target molecule on which the composition or compound exerts its activity can be made using any of a variety of secondary assays described herein.

Although the natural ligand of the TCR is an antigenic peptide associated with a class II MHC molecule on the surface of an antigen presenting cell, T cell activation can be mimicked in a variety of ways, including by cross-linking or binding the TCR with an anti-TCR antibody, by cross-linking or binding CD3 which becomes associated with TCR during activation, by exposing T cells to phorbol myristate acetate (PMA) which stimulates the activity of two groups of the of the protein kinase C (PKC) family, and/or by exposing cells to a calcium ionophore (such as ionomycin or A23187) which increases intracellular Ca^{+2} which is required for calcineurin to de-phosphorylate NF-AT (see, for example, Flanagan et al., *Nature, 352*: 803 - 807 (1991)). The ability to activate T cells without the necessity of either using antigen presenting cells or providing soluble class II MHC peptide complexes is an attractive feature for designing a rapid screen for compounds that affect T cell activation. However, one drawback of using the chemical mimics PMA and a calcium ionophore, such as ionomycin, to activate T cells is that these compounds activate T cells by stimulating intracellular enzymes that are involved in relatively late steps along the signaling cascade. In particular, such "late step activation" bypasses the portion of the signaling cascade upstream of PKC and the calcium released from intracellular storage which is more closely associated with the transmembrane α and β chains of TCR and the associated CD3 complex and which includes the participation of a variety of other enzymes such as the src family of kinases (such as fyn, lck) and the ZAP-70 kinase involved in early steps of the T cell activation (see, Figures 1 and 2). Accordingly, in the high-throughput, primary screen described herein, activation is preferably initiated by cross-linking TCR, or the associated CD3 complex present on the surface of T cells, thereby retaining more of the physiological components of the signaling cascade as potential targets at which an immune regulator may act.

Preferably, in the primary screen described herein, T cell activation is initiated by using an antibody to the α and/or β chains of TCR. Either a Vα or Vβ-specific antibody or a "Pan-αβ" antibody that binds to the constant regions of the α and/or β proteins of all human TCR molecules would be especially convenient for initiating T cell activation in the primary screen described herein. In a preferred embodiment described below, the anti-TCR monoclonal antibody (MAb) 16G8 (Endogen, Cambridge, MA), which specifically binds the Vβ8.1 region of the TCR expressed on Jurkat cells (ATCC Accession Number TIB152), is used to initiate T cell activation.

Whether a particular anti-TCR or anti-CD3 antibody is capable of activating T cells

can be readily determined by using any of a variety of assays for T cell activation. For example, after incubating T cells with a particular anti-TCR or anti-CD3 antibody, the T cells can be assayed for secretion of a cytokine, such as IL-2, into the surrounding medium which is indicative of T cell activation. Such an assay can be routinely performed by using an antibody specific for the cytokine in a standard enzyme linked immunosorbent assay (ELISA). For example, antibodies specific for IL-2 and for IL-4 are commercially available (Endogen, Cambridge, MA; Boehringer Mannheim, Indianapolis, IN).

Alternatively, whether a particular anti-TCR or anti-CD3 antibody can activate T cells can be determined using a T cell line transfected with a recombinant reporter gene, such as the luciferase (LUC) or β-galactosidase (LAC) genes, placed under the control of an enhancer sequence from a cytokine gene involved in T cell activation. Examples of such a recombinant reporter gene construct is found on the IL-2LUC plasmid in which transcription of the luciferase gene is under the control of the IL-2 promoter/enhancer sequence or the NFATLUC plasmid in which transcription of the luciferase gene is under the control of NF-AT binding sequences of the IL-2 gene (Northrop et al., *J. Biol. Chem., 268*: 2917 - 2923 (1993) and see below). Thus, in T cells transfected with such recombinant reporter genes, the expression of the reporter gene will only be observed if the anti-TCR or anti-CD3 antibody actually activates the T cell.

Still another routine way to assay for an antibody's ability to activate T cells is to use a proliferation assay. For example, in one common version of a proliferation assay, peripheral blood lymphocytes (PBLs) containing T cells are incubated with the antibody (which specifically reacts with T cells in the PBL mixture) for 48 to 72 hours, after which the cells are pulse-labeled with ^{3}H-thymidine for an additional 24 hours. Cells are then harvested on glass filters and counted in a scintillation counter for incorporated ^{3}H-thymidine as evidence of growth of the activated T cells in the PBL mixture. Proliferation assays are most useful and convenient for determining the ability to activate T cells when the antibody is capable of binding TCR molecules on a broad range of T cells, such as "pan-αβ" antibodies mentioned above, which are capable of activating enough T cells to proliferate and be detected in the PBL mixture.

The primary screen for immune regulators of T cell activation described herein comprises a multi-step, high-throughput screening procedure which utilizes at each step a cell-based assay so that candidate compositions or compounds that exhibit an immune

regulatory activity are likely to bind to extracellular targets on the surface of a T cell or be capable of traversing the T cell's membrane to reach intracellular targets.

The T cells used in each step of the primary screen are transfected with recombinant plasmids containing a reporter gene, such as the luciferase gene (LUC). Expression of the reporter gene product, or lack of expression, is indicative of the ability of a composition or compound to regulate T cell activation. The use of a reporter gene, such as LUC, has the added feature that its gene product, luciferase, is well characterized and easily detected with a high signal-to-noise ratio, making the assay steps of the screen highly sensitive. In addition, a luciferase assay can be performed routinely in standard 96-well plates, which permits the efficiency of multi-channel pipetting and robotics. Furthermore, the screen may be used for detecting both stimulators and inhibitors of T cell activation.

In each of the steps of this screen, activation of the T cells usually takes 4 to 8 hours to reach plateau levels of the reporter gene activity, and therefore, the steps of the screen can be performed in a single day or with an overnight incubation. However, upon incubations of greater than 8 hours, luciferase levels begin to decline due to apoptosis. Therefore, as explained below, the immune stimulators identified in the primary screen should be further assayed for anti-apoptotic activity. In the primary screen, the sensitivity of the screen for compositions or compounds that are immune stimulators can be increased by using a sub-optimal TCR stimulus so that an increased reporter gene expression is more readily detected compared to control cells that are not exposed to the composition or compound being tested. A sub-optimal T cell activation can be achieved, for example, by using a more dilute concentration of an anti-TCR antibody than is normally used to obtain a maximal activation of the T cells used in the screen. Such sub-optimal T cell activation conditions not only increase the sensitivity of the screen to stimulatory compositions or compounds, but still provides a sufficient level of reporter gene expression to screen for compositions or compounds having inhibitory activity.

Primary Screen for Immune Regulators

The primary screen for immune regulators of T cell activation comprises three assays (steps). A diagram of an example of a primary screen of this invention is depicted in Figure 3. Each assay is a cell-based assay using a T cell line transfected with a recombinant plasmid containing a reporter gene, more particularly the structural coding sequence from the reporter gene, operatively linked to a promoter/enhancer sequence of a cytokine gene involved in T

cell activation, such that reporter gene expression is under the direct control of the cytokine gene promoter/enhancer sequence. When a signal for T cell activation is transduced to the transcription control factors that bind to the cytokine promoter/enhancer sequence, the reporter gene is expressed. Preferably, the reporter gene is the luciferase gene and the T cells are the human lymphoma T cell line Jurkat which express a TCR having a Vβ8.1 protein. In addition, the exact order in which the three assays of this primary screen are performed is not critical, because the pooling of the data from all three assays enables one to determine whether a composition or compound has a significant immune inhibitory or stimulatory activity. However, in a more preferred embodiment, the assays of the primary screen are performed in the order listed below which permits a large number of compositions or compounds to be routinely and efficiently screened (high-throughput) with an economical use of resources (for example, time, reagents, cells).

Step 1. Activation Assay

The first step of this multi-step primary screen for immune regulators uses a human T cell line transfected with a plasmid containing the structural coding sequence of a reporter gene, such as the luciferase gene (LUC) (see, De Wet et al., *Mol. Cell. Biol., 7*: 725 - 737 (1987) (luciferase cDNA and 5' and 3' flanking sequences at Figure 1, page 728); see also GenBank/EMBL Accession Number X65323, luciferase structural coding sequence at nucleotides 76 to 1725) operatively linked to the promoter/enhancer transcription control region of a cytokine gene involved with early activation of T cells, such as interleukin-2 (IL-2) (see, GenBank Accession Number J00264, complete sequence for human IL-2 gene), such that transcription of the reporter gene is under the exclusive control of the promoter/enhancer region sequence. An example of a IL-2 promoter/enhancer region operatively linked to the luciferase structural coding sequence which can be used in this first assay step has previously been described (see, Northrop et al., *J. Biol. Chem., 268*: 2917 - 2923 (1993) (an IL-2LUC plasmid); see, also, Holbrook, et al., *Nucleic Acids Res., 12*: 5005 - 5013 (1984) and Durand et al., *Mol. Cell. Biol., 8*: 1715 - 1724 (1988) (features of IL-2 promoter/enhancer region)). The IL-2LUC plasmid has been deposited in the American Type Culture Collection (ATCC, Rockville, MD) in accordance with the Budapest Treaty (ATCC Accession Number CRL-12091). When the transfected T cells are not activated, the basal level of expression of the reporter gene should be relatively low, preferably at or below the level of detection. The luciferase reporter gene has such a property when placed under the transcriptional control of

the IL-2 promoter/enhancer region. Upon activation via TCR binding or cross-linking, the luciferase activity typically increases 50- to 100-fold, consistent with the level of expression of the native IL-2 gene typically observed in activated T cells.

In this step, transfected T cells are preferably activated by binding TCR, for example using an anti-TCR antibody such as the 16G8 anti-TCR monoclonal antibody (MAb) (Endogen, Cambridge, MA) directed against the human Vβ8.1 variable region of TCR present on Jurkat cells. Typically, this assay is run by immobilizing the antibody on the bottom of wells of a 96-well assay plate or added as a soluble component of the culture medium, then adding T cells, which have been transfected with a plasmid containing the reporter gene under the control of a cytokine promoter/enhancer region, in the presence or, for control cells, the absence of a compound being screened. Other controls recommended for this step include non-activated cells, for example, cells in wells not containing antibody, and validation controls, such as cells activated in the presence of a compound with known inhibitor activity such as cyclosporin A or FK506. Activation of T cells by antibody typically takes 4 to 8 hours, after which time the plates can be assayed for reporter gene expression, although the plates may also be incubated longer, for example overnight particularly to identify anti-apoptotic agents (see below), and assayed the next day. If the reporter gene is luciferase, standard luciferase measurements can be efficiently run using multi-pipetting (manual or automated) and an automated plate reader such as a luminometer (Packard Top-Count, Canberra, Australia). The level of luciferase expression relative to control cells is a direct indication of each compound's ability to further stimulate or to inhibit cytokine gene expression and, therefore, T cell activation.

As noted above, the sensitivity of this step for identifying and quantifying a composition's or compound's immune stimulatory activity can be enhanced by using conditions that provide a sub-optimal level of T cell activation so that an increase in the expression of a reporter gene is more easily detected. Such a sub-optimal level of T cell activation can be effected by using, for example, a lower concentration of anti-TCR antibody than required for maximal reporter gene expression. A sub-optimal level of T cell activation still permits the assay to be used for identifying compositions or compounds with inhibitory activity since a decrease in reporter gene expression can still be detected.

Accordingly, this first assay step distinguishes candidate immune stimulators and inhibitors from compositions or compounds that have neither activity. Because the level of

expression of reporter genes, such as the luciferase gene, can be easily quantitated, this first step can also be used to distinguish between immune regulators of varying strengths of stimulatory or inhibitory activity.

Step 2. Toxicity Assay

The Activation Assay, described above, provides the first indication that a composition or compound may be capable of regulating (either stimulating or inhibiting) cytokine gene expression. However, alone, the first step cannot indicate whether the observed regulatory activity of a composition or compound is specific for genes involved in T cell activation or whether the activity of the composition or compound is non-specific so that stimulation or inhibition of the expression of other genes can occur. Such non-specific activity is undesirable because it would threaten to disrupt expression of those genes ubiquitously expressed in all or most cells, i.e., which are necessary to maintain cell viability, and because it causes expression of genes coding for products that may be toxic or oncogenic. Accordingly, a Toxicity Assay step is run, in the same manner as the Activity Assay, but using T cells transfected with a plasmid containing a reporter gene, such as luciferase, operatively linked to the promoter or promoter/enhancer sequence from a gene that is ubiquitously expressed, or at least expressed in a majority of cells of the human body. A preferred promoter/enhancer sequence is from the β-actin gene, an example of a so-called "house-keeping gene" which is expressed in most cells (see, for example, Ng et al., *Mol. Cell. Biol., 5*: 2720 - 2732 (1985) (sequences from β-actin promoter/enhancer); GenBank Accession Number Y00474 (disclosing 2011 nucleotides of human β-actin promoter/enhancer); Gunning et al., *Proc. Natl. Acad. Sci. USA, 84*: 4831 - 4835 (1987) (plasmid containing human β-actin promoter/enhancer)). As in the Activity Assay, the transfected T cells are activated, but the level of expression of the reporter gene in the activated T cells now reflects the expression of genes, such as β-actin, that are expressed in most or all cells, not the expression of cytokine genes involved in early T cell activation. A composition or compound that affects (stimulates or inhibits) the expression of the reporter gene in both the Activation Assay and Toxicity Assay steps performed with a relatively short (i.e., less than 8 hours) incubation period has a high probability of similarly affecting the expression of many genes in the body, including several whose functions are required ubiquitously. For assays run with incubation periods of greater than 8 hours (as in overnight incubations), stimulators of the Activation and Toxicity Assays can be further screened for

anti-apoptotic activity as explained below. In a preferred embodiment, the β-actin promoter/enhancer sequence is operatively linked to the luciferase structural coding sequence, such as in plasmid β-actinLUC deposited in the American Type Culture Collection (Rockville, MD) in accordance with the Budapest Treaty (ATCC Accession Number 98036). Accordingly, the Toxicity Assay step of this primary screen provides a sensitive assay for screening out those compositions or compounds that have a high probability of being generally toxic to cells, owing to their relatively non-specific ability to inhibit or stimulate gene expression and, therefore, as not having a desirable specific T cell regulatory activity.

The ability of this cell-based Toxicity Assay to accurately detect a cytotoxic composition or compound is generally comparable to those cytotoxicity assays which detect cell death based on the loss of an ability to exclude dyes (such as trypan blue) or to reduce the dye MTT (see, for example, Burres et al., *J. Antibiotics, 48*: 380 - 386 (1995)). Comparable results have been obtained in monitoring cytotoxicity using a commercial MTT dye reduction-based assay (Promega, Madison, WI) and a Toxicity Assay of the primary screen of this invention. Unlike cytotoxicity assays based on dye exclusion or reduction, the Toxicity Assay of the primary screen of this invention specifically indicates whether a composition or compound capable of activating or inhibiting cytokine gene expression (Activation Assay) also has the undesirable ability to interfere with the transcription of genes necessary for cell viability.

Step 3. Specificity Assay

Compositions or compounds that appear to regulate cytokine gene expression (as indicated by the Activation Assay step) without being generally toxic to most cells (as indicated in the Toxicity Assay step), can be assayed to determine whether a candidate immune regulator specifically affects the action of a particular cytokine transcription factor, such as NF-AT, NF-κB, AP-1, or octamer-binding complex (Oct), by using a particular regulatory region of the IL-2 enhancer which is known to be specifically bound or otherwise critical for the activity of each of the individual transcription factors. Within the published IL-2 promoter/enhancer sequence (GenBank Accession Number J00264, containing nucleotides 1 - 5737), the sequences specifically bound by a number of key transcription control factors are known: for the NFAT transcription control factor, the sequence is at nucleotides 146 to 175 (Verweij et al., *J. Biol. Chem., 265*;: 15788 - 15795 (1990)) for the NFκB transcription control factor, the sequence is at nucleotides 221 to 244; for the AP-1

transcription control factor, the sequence is at nucleotides 270 to 294; and for the Octamer binding complex (Oct) transcription control factor, the sequence is at nucleotides 337 to 365. For this step, the upstream transcription control region of a reporter gene, such as the luciferase gene, is replaced with multiple repeats of the individual factor binding sites. For example, for determining whether a candidate composition or compound specifically affects NFAT-mediated transcription of genes involved in T cell activation, three repeats of the NFAT binding sequence from the IL-2 promoter/enhancer region can be operatively linked to the luciferase structural coding sequence and used to determine whether a candidate composition or compound specifically affects NFAT (see, Northrop et al., *J. Biol. Chem.*, *268*: 2917 - 2923 (1993) (NFATLUC plasmid)). A similar construct but having three repeats of the NFκB binding sites (instead of NFAT binding sites) of IL-2 promoter/enhancer region operatively linked to the luciferase structural coding sequence has also been described (NFκBLUC plasmid, see Northrop et al., 1993) and would be useful in this step for determining whether a candidate immune regulator specifically affects NFκB-mediated gene expression. Operatively linking multiple repeats of a particular transcription factor binding sequence to the structural coding sequence of a reporter gene generally provides a highly sensitive assay for determining whether a candidate regulator composition or compound specifically affects transcription mediated by the particular transcription factor.

Some transcription factors may work cooperatively to stimulate cytokine gene transcription. This assay, alone, does not distinguish between a candidate immune regulatory composition or compound that affects a cooperatively acting transcription factor and a candidate immune regulatory composition or compound that affects a transcription factor that is not involved in cooperative binding. Nevertheless, because this Specificity Assay step employs discrete sequences known to be involved in transcription regulation of a cytokine gene, such as IL-2, if the candidate composition or compound causes an effect (stimulatory or inhibitory) on reporter gene expression, then the possible targets at which the candidate regulatory composition or compound acts will be rapidly narrowed to those factors whose control over cytokine gene transcription requires the particular sequence used in the plasmid construct.

As noted above, this assay step can be conducted under sub-optimal conditions for T cell activation to increase the sensitivity of the assay for detecting compositions or compounds that stimulate T cell activation.

Overnight T Cell Activation and Primary Screen Assays

The use of overnight incubations permits additional samples to be assayed overnight in the absence of supervising personnel. However, the Jurkat cell line is susceptible to T cell antigen receptor activation-dependent apoptosis and this fact must be taken into consideration for at least some applications of the primary screen of this invention. Apoptosis during activation of T cells probably occurs by means of the fas - fas ligand (fasL) pathway in which Jurkat cells, which constitutively express fas, upregulate fasL upon activation via the T cell antigen receptor (Dhein et al., *Nature, 373*: 438 - 441 (1995); Brunner et al., *Nature, 373*: 441 - 444 (1995); Ju et al., *Nature, 373*: 444 - 448 (1995)). However, the kinetics of this induction of apoptosis are delayed with respect to the transactivation of genes under the control of the IL-2 promoter/enhancer, such as in an IL-2LUC reporter gene construct. Thus, during activation of a Jurkat cell transfected with an IL-2LUC reporter gene construct, luciferase levels will rise for the first 4 to 8 hours and thereafter begin to decline thereafter. Compositions or compounds which inhibit luciferase expression from the IL-2LUC reporter gene construct are generally unaffected by longer periods of activation, i.e., even if apoptosis occurs, an inhibition of luciferase expression can still be detected as a failure to induce expression of the reporter gene product, such as luciferase. Thus, the use of the primary screen of the invention for inhibitors of T cell activation may be performed using incubation periods longer than 8 hours.

However, apoptosis in Jurkat cells that are activated for greater than 8 hours (as in overnight incubations periods which are typically 12 to 16 hours) will affect the screen for compositions or compounds that stimulate T cell activation. Compositions or compounds which are identified as putative stimulators of T cell activation in the Activation Assay of the primary screen using activation periods of greater than 8 hours should be further screened in a modified Toxicity Assay in which the compositions or compounds are tested for the ability to stimulate reporter gene expression in resting (no T cell activation) cells and in cells activated for greater than 8 hours. This combination of Activation Assay and modified Toxicity Assay can be used to identify anti-apoptotic agents in arrays or libraries of various compositions or compounds. Thus, if the Toxicity Assay of the primary screen uses Jurkat cells transfected with a β-actinLUC reporter gene construct as described above, then the compositions or compounds should be tested for the ability to stimulate luciferase activity in the transfected Jurkat cells in the absence (i.e., no activation) and presence of prolonged (greater than 8

hours) activation conditions. Compositions or compounds which do not stimulate luciferase expression in both resting and activated Jurkat cells (negative for the modified Toxicity Assay) are classified as specific immune activators or stimulators of IL-2 transcription and should be screened in the Specificity Assay and further characterized using one or more Secondary Assays described below. Compositions or compounds which stimulate luciferase expression in the Jurkat cells under non-activating conditions are classified as non-specific stimulators or activators of gene transcription (positive for the Toxicity Assay) and are discarded. Compositions or compounds which stimulate luciferase expression in the Jurkat cells containing the β-actinLUC reporter gene construct which were activated overnight (i.e., greater than 8 hours), but not in non-activated resting cells, are likely to be inhibitors of apoptosis, i.e., anti-apoptotic agents. Anti-apoptotic activity of such compositions or compounds can be confirmed and further characterized using one or more additional apoptosis assays (see Secondary Assays, below).

Anti-apoptotic agents identified by the screening protocol described herein may find therapeutic utility in treating infectious diseases, including acquired immune deficiency syndrome (AIDS) where apoptosis of responding T cells has been correlated with a weakened immune response (Thompson, *Science, 267*: 1456 - 1462 (1995)).

Secondary Assays for Target Identification

After the primary screen of this invention has identified a candidate composition or compound as an immune regulator, a number of secondary assays are available that can be performed to more fully characterize the intracellular target of the composition or compound and its mode of action in the T cell activation signaling cascade (Figures 4 and 5). The following is a list of some of the various secondary assays that can be used to further characterize compositions or compounds identified as immune regulators in the primary screen.

1. Additional Specificity Assays

After the Specificity Assay of the primary screen, additional specificity assays may be run using analogous constructs differing only in the specific transcription factor sequence operatively linked to the reporter gene. For example, after screening with the NFATLUC plasmid in the Specificity Assay, it may be useful to test the candidate immune regulatory composition or compound in cells transfected with analogous NF-κBLUC, AP-1LUC, and/or OctLUC plasmids (construction of AP-1LUC in Examples below).

2. Ca^{++} Ionophore/PMA Activation Assay

This assay is designed to determine whether the target of an immune inhibitor identified in the primary screen is located in the signaling cascade upstream or downstream of the PKC (1 in Figure 1) and calcium ion (2 in Figure 1) and calcium-dependent (such as calcineurin, 3 in Figure 1) steps of the signaling cascade in the T cell. This assay uses T cells transfected with the same plasmid constructs used in step 3 (Specificity Assay) of the primary screen, but uses chemical mimics for T cell activation, such as PMA and a calcium ionophore, such as ionomycin, to activate the T cells. PMA acts on two groups of protein kinase C (PKC) to initiate a signal for T cell activation, and ionomycin acts to increase the calcium ion concentration thereby stimulating calcineurin activity in the signaling cascade (see, Figure 1). The location of PKC and calcineurin are relatively well established in the signaling cascade. Accordingly, a composition or compound that inhibited reporter gene expression in the Specificity Assay of step 3 of the primary screen, but not in this assay where signaling starts at PKC and calcium, most likely acts on a molecule that is involved in a step of the signal cascade located upstream of PKC and calcineurin; such as ZAP-70 (4 in Figure 1). In contrast, a composition or compound that inhibits reporter gene expression in the Specificity Assay of the primary screen and also in this assay, where T cell activation is initiated with PMA and a calcium ionophore most likely acts on a molecule involved in a step of the signaling cascade located downstream of PKC and calcium.

3. Calcium Ion Flux Detection Assay

This assay measures increases in intracellular calcium ion upon crosslinking of the TCR. A composition or compound identified in the primary screen as a stimulator of T cell activation may potentiate calcium ion flux under conditions of sub-optimal TCR signal. Compositions or compounds which augment calcium ion flux can be identified using the fluorescence calcium ion stain Indo 1 (Molecular Probes, Eugene, Oregon). In this assay, cells are pretreated with the dye, then exposed to an anti-TCR crosslinking agent in the presence or absence of a test composition or compound. Calcium ion flux is quantitated using a fluorescent plate reader dividing readings at 405 nm (calcium ion bound Indo 1 maximum) by 525 nm (unbound Indo 1 maximum) (see, for example, Viola et al., *Science, 273*: 104 - 106 (1996)).

4. Gelshift Assay (Electrophoretic Mobility Shift Assay (EMSA))

This *in vitro* assay is used to determine whether a composition or compound is capable of inhibiting the actual binding of a transcription control factor to its cognate binding sequence in the promoter/enhancer region of a cytokine gene involved in early activation of T cells. Preferably, the promoter/enhancer region is from the IL-2 gene (Holbrook, et al., *Nucleic Acids Res., 12*: 5005 - 5013 (1984)). This assay is based on the phenomenon that the position at which a particular DNA molecule migrates in a gel during electrophoresis is shifted when a transcription factor binds its cognate DNA sequence present in that DNA molecule. Typically, the protein-DNA complex migrates with a slower mobility than unbound DNA. Thus, if a composition or compound identified in the primary screen disrupts the binding of a transcription factor to its cognate DNA binding sequence then there should be a detectable change in the relative amounts of protein-DNA complex (at the slower mobility position in the gel) and unbound DNA (at a faster position in the gel). This may reflect a direct inhibition of the DNA-binding ability or a lack of proper cellular localization of a transcription factor. Alternatively, a composition or compound identified in the primary screen as an immune stimulator may potentiate binding of a transcription factor under conditions of sub-optimal TCR signaling.

5. NFATp Dephosphorylation Assay

This assay is designed to identify the mode of action of an immune inhibitor identified in the primary screen with respect to the phosphorylation state of NFATp. In particular, this assay determines whether an immune inhibitor affects the dephosphorylation of the NFATp protein (5 in Figure 1), which is characteristic of an inhibitor acting at calcineurin (3 in Figure 1). The immune regulators cyclosporin A and FK506, for instance, have such an activity (reviewed in Schreiber and Crabtree, *Immunol. Today*, 13: 136 - 142, 1992) and are generally toxic to many cells, particularly cells of the liver and kidneys. Accordingly, this NFATp dephosphorylation assay aims at excluding compositions or compounds that inhibit calcineurin and would therefore have a similar toxicity as cyclosporin A or FK506.

As noted above, NFATp (5 in Figure 1) is a member of a family of transcription factors involved in T cell regulation (reviewed in Rao, *Immunol. Today, 15*: 274 - 281 (1994)). NFATp consists of 890 amino acids and runs with an apparent molecular weight (Mr) of 130,000 by SDS-PAGE (McCaffrey et al., *Science, 262*: 750 - 754 (1993)). Treatment of purified NFATp with calcineurin (3 in Figure 1), a serine/threonine protein

phosphatase, or activation of T cells leads to a decrease in its apparent Mr in the absence of cyclosporin A owing to the dephosphorylation of the protein (6 in Figure 1; see, Liu, *Immunol. Today, 14*: 290 - 295 (1993); Jain et al., *Nature, 365*: 352 - 355 (1993); Park et al., *J. Biol. Chem., 270*: 20653 - 20659 (1995)).

Detergent lysates are prepared from activated or non-activated T cells in the absence or presence of compositions or compounds identified from the primary screen. NFATp is immunoprecipitated from the lysates with a specific anti-NFATp-antibody, run on a SDS-polyacrylamide gel (standard SDS-polyacrylamide gel electrophoresis), and transferred to a nitrocellulose membrane for immunoblot (Western blot) analysis using the same anti-NFATp antibody. Inhibition of calcineurin can be determined by examining the apparent mobility of NFATp. A composition or compound that inhibits calcineurin would prevent dephosphorylation of NFATp. A composition or compound identified as an immune stimulator in the primary assay that activates calcineurin when calcium ion is limiting would potentiate the dephosphorylation of NFATp, augmenting its transcriptional activity.

6. Phosphotyrosine Proteins Assay

This secondary assay is recommended for compositions or compounds which inhibited reporter gene expression in the Specificity Assay (step 3) of the primary screen but did not inhibit reporter gene expression in PMA/ionophore-activated T cells in the Ca^{+2} Ionophore/PMA Activation Assay (secondary assay 2, above). This assay is designed to detect changes in the pattern of tyrosine phosphorylated proteins in T cells activated with an anti-TCR antibody and gives a better indication of the point in the signaling cascade at which a composition or compound identified in the primary screen asserts it affect. It is well established that protein tyrosine kinases are critically involved in a variety of signal transduction pathways. One group of protein kinases consists of receptor tyrosine kinases whereas the other group contains the non-receptor type kinases (reviewed in Hunter and Cooper, *Ann. Rev. Biochem., 54*: 897 - 930 (1995) and Fantl et al., *Ann. Rev. Biochem., 62*: 453 - 481 (1993)). Protein tyrosine kinases are crucial for the signal transduction through lymphocyte antigen receptors (reviewed Weiss and Littman, *Cell, 76*: 263 - 274 (1994)). Anti-phosphotyrosine antibodies have proved to be extremely valuable for the evaluation of signaling events (Friedman et al., *Proc. Natl. Acad. Sci. USA, 81*: 3034 - 3038 (1984); Daniels et al., *Proc. Natl. Acad. Sci. USA, 82*: 2684 - 2687 (1985)).

In this assay, detergent lysates are prepared from activated or non-activated T cells, in

the absence or presence of lead compositions or compounds; the lysates electrophoresed on SDS-gels and transferred to a nitrocellulose membrane for Western blot analysis using anti-phosphotyrosine antibodies. Changes in the pattern of tyrosine phosphorylated proteins in the presence of a candidate composition or compound indicate a mode of action closer to the TCR and before the PKC and calcium-dependent steps of signal transduction (for example, ZAP70, lck, lyn in Figure 1). A composition or compound identified as an immune stimulator in the primary assay may augment tyrosine phosphorylation under conditions of sub-optimal TCR signaling.

7. Assay for pp120 Dephosphorylation

The purpose of this assay is to determine whether the mode of action of compositions or compounds identified in the primary screen affects tyrosine phosphorylation in HUT78 T cells stimulated with PMA and ionomycin. In particular, a protein (pp120), which has an apparent molecular weight (Mr) of 120,000 daltons, becomes phosphorylated on tyrosine upon activation with ionomycin. This reaction is distinct from the phosphotyrosine protein profile in stimulated T cells measured in the Phosphotyrosine Protein Assay described above. The phosphorylation of pp120 is inhibited in HUT78 T cells activated in the presence of cyclosporin A. Accordingly, this assay provides a comparison of the mode of action of a candidate composition or compound identified in the primary screen and cyclosporin A, which is known to inhibit calcineurin. The assay is run in basically the same manner as the Phosphotyrosine Protein Assay described above, except the HUT78 T cells are activated with PMA and ionomycin.

8. Phosphorylation Assay of Nuclear cdc2 and cdk2

The purpose of this assay is to determine whether compositions or compounds identified in the primary screen affect the phosphorylation state of the cyclin-dependent kinases (cdks) cdc2 and cdk2 in the nucleus (see Figure 1). The cyclin-dependent kinases are a class of serine/threonine kinases that regulate cell cycle checkpoints and progression through the cell cycle. Cdk activity is tightly regulated by several mechanisms, including an association with a specific cyclin regulatory subunit, activating and inhibiting phosphorylations, and binding of a class of small proteins termed the cyclin kinase inhibitors (see, Morgan, *Nature, 374*: 131- 134 (1995)). The kinase activity of cdc2 is required for the G2 to M phase transition of the cell cycle (see, Nurse, *Nature, 344*: 503 - 507 (1995); Norbury et al., *Ann. Rev. Biochem., 61*: 441 - 470 (1992); Atherton-Fessler et al., *Seminars*

Cell Biol., 4: 433 - 442 (1993)). The kinase activity of cdk2 is required in late G1 phase of the cell cycle for transition into S phase, and during the S and G2 phases of the cell cycle (Gu et al., *EMBO J., 11*: 3995 - 4005 (1992); Guadgno et al., *Cell, 84*: 73 - 82 (1996)). The inhibitory phosphorylations on both cdc2 and cdk2 occur on a threonine residue at amino acid position 14 (Thr-14) and a tyrosine residue at amino acid position 15 (Tyr-15), and full activation of kinase activity is dependent on the dephosphorylation of both of these residues by the cdc25 dual-specificity phosphatases (see, Gautier et al., *Cell, 67*: 197 - 211 (1991); Galaktionov et al., *Cell, 67*: 1181 - 1194 (1991); Hoffmann et al., *EMBO J., 13*: 4302 - 4310 (1994); Jinno et al., *EMBO J., 13*: 1549 - 1556 (1994)). Nuclear extracts of T cells contain tyrosine-phosphorylated fractions of cdc2 and cdk2, and T cell activation slightly increases the extent of tyrosine phosphorylation. A more detailed description of this assay follows.

To run this assay, typically approximately 100 x 10^6 T cells are pre-incubated for 15 minutes at room temperature in the absence or presence of a composition or compound (for example, 30 μg/ml) identified in the primary screen, followed by activation either with 10 μg/ml anti-T cell receptor antibody (for example, 16G8, Endogen, Cambridge, MA) and 20μg/ml goat-anti-mouse antibody or with 2 μM ionomycin in DMSO (Calbiochem, San Diego, CA) and 50 nM PMA (phorbol-12-myristate-13-acetate) in ethanol (Calbiochem) for 5-10 minutes at room temperature. After a wash with ice-cold PBS (phosphate buffered saline), the cells are lysed in Cytb buffer (10 mM HEPES (pH 7.3), 15 mM KCl, 2.5 mM $MgCl_2$, 0.25% NP-40, 0.1 mM EDTA, 20 μM $ZnCl_2$, 25 mM NaF, 10 mM pyrophosphate, 500 μM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml pepstatin, 50 μg/ml trypsin inhibitor from soybean). The supernatant after centrifugation (for example, 1 min. at 5,000 rpm in an Eppendorf tabletop centrifuge) yields the "cytosolic extract". The pellet is washed with Cytb minus NP40 buffer (Cytb buffer as described above, but with NP-40 omitted) and then extracted with 420-buffer (Cytb-buffer plus 10% glycerol and 420 mM NaCl) for 30 minutes on a rocker at 4°C. The supernatant after centrifugation (for example, 12 minutes at 14,000 rpm in an Eppendorf tabletop centrifuge) yields the "nuclear extract". In the case of assaying for phosphorylation of nuclear cdc2, cdc2 is immunoprecipitated from 1 mg of lysate protein with 5 μl of anti-cdc2-antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. In the case of assaying for phosphorylation of nuclear cdk2, cdk2 is immunoprecipitated using an anti-cdk2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Otherwise, the assays are

identical. Standard immunoblotting (Western blot analysis) is performed with the appropriate antibody and anti-phosphotyrosine antibodies.

The extent of phosphorylation of cdc2 or cdk2 in the presence and absence of a composition or compound from the primary screen can be compared to that seen with cyclosporin A and/or FK506 to determine the relative effect, if any, a composition or compound from the primary screen has on the phosphorylation of nuclear cdc2 or cdk2.

9. Raf, MEK, and ERK Activity Assays

These assays determine whether a composition or compound identified in the primary screen may act on Raf, MEK or ERK (see, Figures 1 and 2; see also, Heidecker et al., *Mol. Cell. Biol., 10:* 2503 - 2512 (1990); Crews et al., *Science, 258*: 478 - 480 (1992); Boulton et al., *Cell, 65*: 663 - 675 (1991)). These proteins are modules of a kinase cascade which is critical for transduction of the T cell activation signal through the Ras pathway which occurs downstream of TCR (see, Thomas et al., *Cell, 68*: 1031 - 1040 (1992) (Ras pathway); Weiss and Littman, *Cell, 76*: 263 - 274 (1994) (Ras pathway and T cell activation); Figures 1 and 2).

To run this assay, detergent lysates are prepared from activated and non-activated T cells in the presence or absence of a candidate immune regulator identified in the primary screen. The lysates are then subjected to SDS-PAGE followed by Western immunoblot analysis using commercially available anti-Raf, -MEK or -ERK antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). All three kinases are activated by phosphorylation through upstream kinases. In particular, Raf is activated by phosphorylation through an unidentified membrane-bound kinase on serine and threonine. MEK is activated by phosphorylation by Raf on serine and threonine residues. ERK is activated by phosphorylation by MEK on tyrosine and threonine residues. Such phosphorylation causes a mobility shift of Raf and ERK in SDS-gels which reflects the extent of the activation. Phosphorylation of MEK does not lead to a mobility shift, however, use of a commercially available anti-MEK antibody (sc-219 from Santa Cruz) permits detection of phosphorylated MEK: the antibody binds less tightly to the phosphorylated than the unphosphorylated form. In addition, *in vitro* kinases assays permit a measurement of the extent of activation. The assay which was described for ERK, using myelin basic protein (MBP) as a substrate (Boulton and Cobb, *Cell Regulation, 2*: 357 - 371 (1991)), can be used to measure the activities of Raf and MEK when used with histidine-tagged MEK and histidine-tagged-ERK, respectively.

10. JNK Activity Assay

The purpose of this secondary assay is to determine whether a composition or compound identified in the primary screen acts at the jun N-terminal kinases (JNKs), which play significant roles in signal transduction from the cell surface to the nucleus, particularly in the formation of the AP-1 transcription factor (reviewed in Karin, *Curr. Opin. Cell Biol. 6*: 415 - 424 (1994); Figures 1 and 2). JNKs are important in the signal integration during T cell activation via TCR cross-linking (Su et al., *Cell, 77*: 727 - 736 (1994)) because they are responsible for the phosphorylation of the transcription factors c-jun, TCF, and ATF2, necessary steps in the formation of the active AP-1 transcription factor complex (Derijard et al., *Cell, 76*: 1025 - 1037 (1994)).

To determine JNK activity, detergent lysates are prepared from activated or non-activated T cells in the absence or presence of a candidate immune regulator identified in the primary screen. Such lysates may then be analyzed by using any or all of a variety of assays for JNK activity, such as an *in vitro* kinase assay of immunoprecipitated JNK using GST-jun fusion proteins as substrates, "precipitation/kinase" assays using GST-jun fusion proteins, and "in-gel" assays which allow visualization of jun phosphorylation by JNK (see, Hibi et al., *Genes Dev., 7*: 2135 - 2148 (1993)).

11. Nuclear Translocation Assay

Compositions or compounds identified by the primary screen can also be analyzed for their ability to affect the translocation of proteins involved in the regulation of cytokine transcription from the cytosol into the nucleus, either upon activation (for example, the translocation of dephosphorylated NFAT, 5 and 6 in Figure 1) or after their synthesis in the cytosol (for example, c-fos). Such proteins typically contain short stretches of basic amino acids which act as nuclear localization signals (NLS) (reviewed in Dingwall and Laskey, *Trends Biochem. Sci.*, 16:478-481, 1991). The transport mechanism mediated by nuclear pore complexes (NPC) is fairly well understood (reviewed in Gerace, *Curr. Opin. Cell Biol.*, 4:637-645, 1992). A composition or compound that blocks the translocation into the nucleus of a specific protein involved in T cell activation, such as de-phosphorylated NFAT, would be particularly desirable owing to the critical role NFAT plays in the expression of cytokines, such IL-2, in T cell activation. In contrast, a composition or compound that blocks or inhibits nuclear translocation in general is less desirable as a therapeutic agent, because such a general inhibitor of nuclear translocation may inhibit translocation of proteins that are unrelated to T

cell activation but important for critical functions necessary to most or all cells.

For this assay, cytosolic and nuclear extracts are prepared as in the Nuclear cdc2 Assay, described above. T cells are extracted with a detergent and low salt (for example, 15 mM KCl in Cytb-buffer, see above) buffer to yield cytosolic extracts having intact nuclei, followed by extraction of the nuclei with a high salt (e.g., 420 mM NaCl in 420-buffer, see above) buffer to yield nuclear extracts. Selected target proteins (such as NFAT, ERK, c-fos, c-jun) that form the transcription factors critical to the expression of cytokine genes, such as IL-2, during T cell activation are immunoprecipitated from the extracts using antibodies specific for those target proteins. The immunoprecipitated proteins are then electrophoresed on SDS-gels and transferred to nitrocellulose membranes for Western blot analysis using the appropriate antibodies. The Western blot analysis allows a comparison to be made in the pattern of the specific target proteins in the nuclear extracts of T cells activated in the presence and the absence of a composition or compound from the primary screen to determine whether the composition or compound affects the translocation of any of the specific target proteins involved in T cell activation.

12. Protein Kinase C (PKC) Assay

Members of the protein kinase C (PKC) family play key regulatory roles in a variety of cellular functions (reviewed in Nishizuka, *Nature*, 308:693-698, 1984), including the transduction of a signal for T cell activation from the TCR (see, Weiss and Littman, *Cell, 76*: 263 - 274 (1994)). The structure, function and regulation of various species of PKC have been reviewed (Newton, *J. Biol. Chem., 270*: 28495 - 28498 (1995)).

To run this assay, detergent lysates are prepared from T cells, activated and non-activated, in the absence or presence of a composition or compound for the primary screen. The lysates or species of PKC immunoprecipitated from the lysates are assayed for kinase activity. Addition of a composition or compound from the primary screen to immunoprecipitated PKC from untreated T cells allows an assessment of a direct effect on a PKC kinase activity.

13. fos/jun Family Protein Expression

The purpose of this assay is to determine whether a composition or compound identified by the primary screen can affect (stimulate or inhibit) the expression of genes coding for proteins of the jun and fos families (8 and 9, respectively, in Figure 1). Jun and fos proteins are components of the dimeric AP-1 transcription factor (see, Angel and Karin,

Biochim. Biophys. Acta., 1072: 129 - 157 (1991)). Different AP-1 proteins (for example, c-jun, junB, junD, c-fos, fosB, fra-1, and fra-2) in conjunction with other transcription factors have been shown to confer distinct regulatory effects, ranging from activation to inhibition of cytokine gene transcription (see, Miner and Yamamoto, *Genes Dec., 6*: 2491 - 2501 (1992)).

To run this assay detergent lysates are prepared from T cells that were activated (and those that were not activated) in the absence or presence of a composition or compound identified in the primary screen. Whole cell lysates are prepared and specific antibodies used to immunoprecipitate one or more of the various fos/jun family members. The immunoprecipitates are then assessed by Western blotting using the relevant antibodies (e.g., available from Santa Cruz Biotechnology, Santa Cruz, CA) to identify the pattern of proteins expressed in the T cells to determine whether a composition or compound can stimulate or inhibit fos or jun protein expression.

14. Mouse Splenocyte and Human Peripheral Blood Lymphocyte Proliferation Assay

Compositions or compounds identified in the primary screen as having immune regulatory activity can be assayed for their ability to stimulate or inhibit proliferation of non-transformed cells by examining the effect of such stimulators or inhibitors have on the proliferation of mouse splenocytes or human peripheral blood lymphocytes (PBL). For example, if mouse splenocytes are used, the candidate immune regulatory compositions or compounds are incubated with splenocytes from naive DBA-2 mice, under conditions that activate T cells and thereby promote proliferation, such as incubation in the presence of the presence of the poly-clonal T cell stimulator Concanavilin A (ConA, Calbiochem, San Diego, CA) at one or more concentrations. After the cells are incubated in the presence of a candidate immune regulatory composition or compound, the cells are pulsed labeled with ^{3}H-thymidine and harvested onto glass fiber filters for scintillation counting (Packard, Meriden, CT). Activation or inhibition of proliferation is measured by the extent to which the labeled ^{3}H-thymidine is incorporated into cells in the presence and absence of candidate immune regulatory compositions or compounds.

15. IL-2 ELISA

As an indication of the ability of a composition or compound of the primary screen to stimulate or inhibit T cell activation, supernatants from T cell proliferation cultures, such as from mouse splenocytes or human peripheral blood lymphocytes, as described above, grown in the presence or absence of a candidate immune regulatory composition or compound can

be assayed for the presence of cytokines secreted during T cell activation. For example, the effect of a composition or compound on cytokine IL-2 production can be tested by quantitation using a standard ELISA set up or commercial kit (for example, Endogen, Woburn, MA). Other cytokines whose expression is stimulated by T cell activation, such as gamma interferon, can be similarly quantitated by ELISA using available antibodies and reagents. Furthermore, T cell culture conditions can be modified to yield cytokines such as IL-4 (Litton et al., *J. Immunol. Methods, 175:* 47 - 58 (1994)), which can also be quantitated by ELISA.

16. Apoptosis Assays

Apoptosis or programmed cell death is an active, physiological form of cellular suicide to regulate cell growth and homeostasis. It is executed by a genetically regulated, evolutionarily highly conserved, cell death program that can react to a wide variety of different stimuli. Alterations in cell survival are implicated in the pathogenesis of an increasing number human disorders such as autoimmune diseases, AIDS, cancer, and neuro-degenerative diseases (see, for example, *Science, 267*: 1456 - 1462 (1995)). Apoptosis assays can be used to determine the mode of action of a composition or compound identified in the primary screen with respect to cytotoxicity to T cells. In contrast to the Toxicity Assay of the primary screen, however, these assays are designed to identify a particular form of cell death, i.e., apoptosis, that may be induced by compositions or compounds identified in the primary screen.

Apoptosis may be specifically induced in activated T cells by a composition or compound identified as an immune suppressor in the primary screen. Such activation-dependent apoptosis may be useful to achieve antigen-specific tolerance which would be particularly beneficial to prevent graft rejection in tissue and organ transplant recipients or to treat autoimmune disorders. In order to identify compositions or compounds that induce such activation-dependent apoptosis, assays are performed on activated and unactivated T cells. Compositions or compounds that induce apoptosis in activated but not unactivated T cells are selected as useful immune suppressors.

The ability to induce apoptosis in resting cells (both T cells and non-T cells) is indicative of a condition under which an immune regulator of the primary screen may exert a toxic effect on cells. Induction of apoptosis in resting cells may not be a significant factor at the concentrations used in the primary screen and in therapeutic uses. However, the ability to

induce apoptosis in resting cells may be an ever increasing concern for higher concentrations of such compositions or compounds, as may accumulate during relatively long-term (i.e., months or years) therapeutic uses.

Apoptosis assays can also be used to confirm anti-apoptotic activity of compositions or compounds that are identified as both immune stimulators and anti-apoptotic agents by an Activation Assay and modified Toxicity Assay as described above. In this case, a secondary assay for apoptosis involves inducing apoptosis by incubating T cells with a TCR-crosslinking agent in the presence or absence of a test composition or compound for greater than 8 hours. This activation-dependent apoptosis is mediated by the induction of fasL and its interaction with fas. Thus, compositions or compounds which inhibit either the induction of fasL or apoptotic pathways initiated by fas will be detected.

The apoptosis secondary assays are based on the fact that apoptosis is defined by a characteristic pattern of morphological, biochemical, and molecular changes that are broadly assigned to three stages: early, intermediate, and late apoptosis. Necrotic cell death is morphologically distinct from cell death by apoptosis. For example, necrotic cell death is characterized by early membrane disruption. In contrast, membrane integrity is maintained until the late stage of apoptosis. In addition, one hallmark of the intermediate apoptotic stage is the internucleosomal cleavage of DNA into distinct fragments of 180 to 200 base pairs and multiples thereof by activated endonucleases.

Various assays for apoptosis are known (see, for example, McGahon et al., *Meth.Cell Biol., 46*: 153 - 185 (1995); Martin et al., *J. Exp. Med., 182*: 1545 - 1556 (1995)). One example of an apoptosis assay is based on labeling (i.e., "staining") of the surface of cells that are undergoing apoptosis compared to cells that are not. A distinct feature of early apoptosis is the specific translocation of the membrane phospholipid phosphatidyl serine (PS) from the inner leaflet (i.e., the cytoplasmic side) of the plasma membrane to the outer surface of the membrane, thereby exposing PS on the cell surface. To date, all apoptosis-inducing stimuli are known to produce this translocation of PS. Cells in early apoptosis can be identified by flow cytometry after staining with fluorescein isothiocyanate (FITC)-labeled Annexin V, which is a 38 kilodalton protein having a high affinity for PS. In order to discriminate against necrotic cell death, which is characterized by early membrane disruption, Annexin V-FITC-labeled cells are also labeled with the DNA intercalating dye propidium iodide (PI). FITC-labeled Annexin V and PI have different detectable fluorescent signals that can be

distinguished from one another. Thus, cells in early apoptosis exclude the PI and only stain positive for Annexin V-FITC, whereas necrotic cells stain positive for both fluorescent markers.

As noted above, distinct fragments of DNA of 180 to 200 base pairs, and multiples thereof, are formed by the internucleosomal cleavage of DNA that occurs in the intermediate stage of apoptosis. Such DNA fragments form a characteristic series of regularly spaced molecules or "DNA ladder" which is observable after electrophoresis through a gel. Thus, in another assay for apoptosis, DNA isolated from cells exposed to known or putative apoptosis stimuli is electrophoresed through a gel, such as agarose or polyacrylamide and the like, and the gel examined for the presence or absence of a DNA ladder of cleavage products.

In order that the invention described herein may be more fully understood, the following examples are set forth. It should be understood that these examples are for illustrative purposes and are not to be construed as limiting this invention in any manner.

EXAMPLES

Example 1: Screening A Synthetic Organic Molecule Library for Immune Regulators

Library of Organic Molecules

A library of approximately 8,000 arylidene diamide compounds was screened for immune regulators using the multi-step primary screen described herein. A library of compounds in array form is commercially available from ArQule, Inc. (Medford, MA), under the product designation AN-1001. The synthesis of the compounds of this library is described in the concurrently filed United States Patent Application (U.S. Serial No.08/641,986, filed April 25, 1996), incorporated herein by reference.

Plasmids

An IL-2 Luciferase (IL-2LUC) recombinant reporter gene plasmid containing the structural coding sequence of a luciferase gene (GenBank Accession Number X65323 (nucleotides 76 to 1725); De Wet et al., *Mol. Cell. Biol.*, 7: 725 - 737 (1987) (see, Figure 1 at page 728)) under the transcriptional control of (operatively linked to) the promoter/enhancer region of the IL-2 gene (GenBank Accession Number J00264) was obtained from Gerald Crabtree (The Howard Hughes Medical Institute, Stanford University, Stanford, CA 94305). The plasmid IL-2LUC was described and used in Northrop et al. (1993). Plasmid IL-2LUC

was used in the first step (Activation Assay) of a primary screen for immune regulators of T cell activation according to this invention. A Jurkat cell line stably transfected with plasmid IL-2LUC was deposited with the American Type Culture Collection (ATCC, Rockville, MD) in accordance with the Budapest Treaty on April 26, 1996 and assigned the ATCC Accession Number CRL-12091.

A β-actinLUC plasmid used in the second step (Toxicity Assay) of the primary screen was constructed by Randall Faircloth and Scott Umlauf (Umlauf et al., 1994). The luciferase gene from the pGL2 plasmid (Promega, Madison, WI) was cloned into the HindIII and BamHI sites of plasmid pHβApr-1-neo (Gunning et al., 1987) to form plasmid β-actinLUC in which a promoter/enhancer region of the β-actin gene is operatively linked to the luciferase structural coding sequence so that transcription of the luciferase coding sequence is under the control of the promoter/enhancer region of the β-actin gene. Plasmid β-actinLUC in an *Escherichia coli* bacterial host cell was deposited in accordance with the Budapest Treaty in the American Type Culture Collection (ATCC, Rockville, MD) on April 26, 1996 and assigned Accession Number 98036. For the Toxicity Assay in the primary screen, the β-actinLUC plasmid is most preferably used in transient transfected cell lines since in these lines the basal level of luciferase expression is significantly higher than in stable lines. In the examples herein, the levels of luciferase gene expression were typically 20-fold higher in transiently transfected cells than in stably transfected cells.

An NFATLUC plasmid was obtained from Gerald Crabtree (Northrop et al., 1993) for use in the third step (Specificity Assay) of the primary screen. The NFATLUC plasmid contains three copies of the NFAT binding sequence of the IL-2 promoter/enhancer region (NFAT binds to a sequence defined by nucleotides 146 to 175 of IL-2 promoter/enhancer sequence in GenBank Accession Number J00264) operatively linked to the luciferase structural coding sequence. A Jurkat cell line stably transfected with plasmid NFATLUC was deposited with the American Type Culture Collection (ATCC, Rockville, MD) in accordance with the Budapest Treaty on April 26, 1996 and assigned the ATCC Accession Number CRL-12092.

An AP-1LUC plasmid was constructed that contained the AP-1 binding sequence of the IL-2 promoter/enhancer (AP-1 binds to a sequence defined by nucleotides 270 to 294 of IL-2 promoter/enhancer sequence in GenBank Accession Number J00264) operatively linked to the luciferase structural coding sequence in a manner analogous to the other recombinant

LUC plasmids. In particular, complementary DNA strands containing the multimeric AP-1 binding sequences of the AP-1LUC plasmid (three copies of the AP-1 regulatory sequence of the IL-2 promoter/enhancer) were synthesized by and purchased from Operon Technologies, Inc. (Alameda, CA). These complementary strands were annealed to form a multimeric AP-1 binding site having 5' four-base overhangs of an XhoI restriction endonuclease site for cloning into the XhoI restriction site of plasmid NFATLUC.

The first strand of the synthetic AP-1 binding site containing the three tandem copies of the AP-1 transcription control sequence has the nucleotide sequence of SEQ ID NO:1:
TCG AGA ATT CCA AAG AGT CAT CAG AAG AAA TTC CAA AGA GTC ATC AGA AGA AAT TCC AAA GAG TCA TCA GAA GAC.

The complementary second strand of the synthetic AP-1 binding site containing the three tandem copies of the AP-1 transcription control sequence has the nucleotide sequence of SEQ ID NO:2:
TCG AGT CTT CTG ATG ACT CTT TGG AAT TTC TTC TGA TGA CTC TTT GGA ATT TCT TCT GAT GAC TCT TTG GAA TTC.

The first strand (SEQ ID NO:1) and second strand (SEQ ID NO:2) were annealed and cloned into the XhoI site of plasmid NFATLUC using standard methods (Maniatis et al., Molecular Cloning (A Laboratory Manual), (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982)). Digestion of the plasmid NFATLUC with XhoI permits the insertion of the synthetic oligonucleotide in place of the three tandem copies of the NFAT transcription control sequence.

Transfections

Transfections were performed by electroporation: 250 volts, 960 μF in a 0.4 cm cuvette. Approximately 1 x 10^7 cells were transfected in 0.5 ml. To obtain stably transfected cell lines, the transfected cells were recovered from the electroporation cuvette and grown in suspension in selection medium: RPMI 1640 (GIBCO BRL, Gaithersburg, MD) supplemented with fetal calf serum (10% v/v, Hyclone, Logan, Utah), L-glutamine (2 mM, GIBCO BRL), penicillin/streptamycin (100 U/ml and 100 μg/ml, respectively, GIBCO BRL), 2-mercaptoethanol (55 μM, GIBCO BRL), and geneticin (G418, GIBCO BRL, Gaithersburg, MD) at 1 mg/ml. The vast majority of the cells died during selection and the stable transfectants expanded noticeably after about two weeks. The relatively rapid rate of recovery of the cells from the selection media indicated that the transfected cell lines were

oligo-clonal which reduces the probability of integration position artifacts. Stably transfected cell lines were used in the assays of the first step (Activation Assay) and the third step (Specificity Assay) of this primary screen. No cloning of transfectants was performed.

To obtain transiently transfected Jurkat cell lines for use in the second step (Toxicity Assay) of the primary screen, cells transfected with plasmid β-actinLUC were recovered from the electroporation cuvette and grown in suspension overnight in the liquid medium as described above for the stably transfected cells, but without selection (i.e., the medium was not supplemented with geneticin). Assays were performed within 72 hours of electroporation.

Screening Compounds for Immune Regulatory Activity

Jurkat (human T cell lymphoma) cells were transfected with IL-2 LUC plasmid for use in the first step (Activation Assay), with β-actinLUC plasmid for use in the second step (Toxicity Assay), or with NFATLUC plasmid for use in the third step (Specificity Assay) of the primary screen for immune regulators.

Jurkat T cells were activated using both the 16G8 anti-TCR monoclonal antibody (MAb) (Endogen, Cambridge, MA), which is specific for the human Vβ 8.1 variable region of the β chain of the TCR found on Jurkat cells, and phorbol myristate acetate (PMA, 20 nM, Calbiochem, San Diego, CA). The wells of a 96-well tissue culture plate were coated with the 16G8 MAb (which adhere to the surface of the wells) so that the TCR on the surface of T cells in each well would be crosslinked by the MAb on the surface of the well, thereby simultaneously immobilizing and activating the T cells. The antibody concentration (titer) in the antibody coating solution should be carefully measured. At the optimal antibody concentration (10 μg/ml), the level of activation reflected in luciferase gene expression is 50- to 100-fold greater in activated T cells than in non-activated T cells. To screen libraries of compositions or compounds for both immune inhibitors and activators in the same assay, it is preferable to use a sub-optimal 50% activation (i.e., 25- to 50-fold stimulation). This can be accomplished by using an antibody concentration of approximately 1 μg/ml in PBS (GIBCO BRL). Titering each lot of antibody permits adjustments for lot-to-lot variation. A stock solution of antibody can be reused to coat several plates with antibody since each coating seems to deplete the antibody solution only slightly. Care should be taken, though, to monitor the fold-stimulation of luciferase expression so that potential inhibitors are less likely to be missed during the screening procedure due to insufficient activation of T cells in the

wells.

The luciferase assay is best performed using a luminometer which can read 96-well culture plates, such as the Packard Top-Count (Packard, Meriden, CT). Plates can be obtained which are suitable for tissue culture yet are composed largely of opaque plastic which reduces the background luminescence. The View-Plate from Packard is especially well-suited for this assay since the bottom of the well is clear allowing the operator to view the condition of the cultured cells prior to lysis. Once the antibody coating solution has been removed from the wells and T cells added, a candidate composition or compound can then be added in an aqueous solution. Some concentration of organic solvents can be tolerated at this stage, however the antibody bound to the wells must not be denatured.

The following three-step primary screen was used to screen a library of approximately 8,000 synthesized oxazalone-derivative arylidene diamide compounds of the ArQule, Inc. AN-1001.

For the first step (Activation Assay) of this primary screen, each compound from the library was added to a MAb-coated well in 25 µl of PBS/DMSO (98%/2%). Cells (2 X 10^5) premixed with PMA (20 ng/ml) were added to a final volume of 100 µl. The final concentration of each compound was approximately 20 µM. Routinely, several wells of controls were included: non-activated cells (wells with no antibody), activated cells with no compound, and validation controls such as cells activated in the presence of the immunosuppressive agent cyclosporin A (CsA). The cells were incubated for several hours (at least 4 hours) to allow for peak expression of luciferase. Expression began to plateau after four hours and, typically, remained constant for as long as 8 hours. At the end of the incubation, the plates were centrifuged (1,500 rpm for 7 minutes) and the culture medium was removed. Cells were washed with 1X PBS (GIBCO BRL, Gaithersburg, MD), the plates spun again, and the PBS removed. Since the luciferase is an intracellular protein, the cells were lysed to assay for luciferase activity. The luciferase assay is compatible with many low-concentration detergent lysis buffers including Reporter Lysis Buffer supplied by Promega (Madison, WI). A volume of 20 µl of Reporter Lysis Buffer was sufficient to achieve lysis in two minutes at room temperature. The luciferase substrate luciferin, was then added along with an energy source such as ATP or acetyl-CoA, routinely in a volume of 100 µl. The luciferin-acetyl-CoA combination supplied by Promega (Madison, WI) is preferable since the half-life of decay of luminescence is 5 minutes as compared to one minute for ATP systems.

Plates were counted in a luminometer (light units are counts per second) as quickly as possible since light emission decays logarithmically. Results are shown in Figure 7. For cells transfected with the IL-2LUC plasmid for use in the first step (Activation Assay) of the primary screen: lysates of unstimulated cells exhibited an emission of about 200 light units (medium, Figure 7), lysates of cells stimulated with antibody and PMA exhibited an emission of approximately 1200 light units (TCR, Figure 7), and lysates of cells stimulated in the presence of an inhibitory compound of the library typically exhibited an emission of approximately 800 lights (C4, Figure 7). Compounds that exhibited inhibitory activity were further tested in the next step, the Toxicity Assay.

For the second step (Toxicity Assay) of this primary screen, Jurkat cells were transiently transfected (electroporated) with the β-actinLUC plasmid (see above). Eighteen hours after electroporation, viable cells were plated in the MAb-coated wells and in the presence of the compound to be tested (approximately 20 μM) as described above in the Activation Assay. In this Toxicity Assay, the expression of luciferase in the cells transfected with the β-actinLUC plasmid is constitutive and poorly stimulated by TCR cross-linking in the assay wells. Neither cyclosporin A (CSA) nor the compounds that inhibited the Activation Assay (for example, AT-7 in Figure 8) significantly affected luciferase activity in non-activated cells of this Toxicity Assay. Cyclosporin A (known to be generally toxic to many cells) inhibited luciferase expression by approximately 40% in activated cells. In contrast, none of the compounds that significantly inhibited luciferase activity in the Activation Assay inhibited luciferase activity in this Toxicity Assay. This result suggested that the inhibition of luciferase expression under the regulation of the IL-2 promoter/enhancer region in the Activation Assay (first step) was specific and not of a general (toxic) nature. Accordingly, these compounds were next screened in a Specificity Assay (third step) for their ability to affect luciferase expression under the specific control of the NFAT transcription factor.

For the third step (Specificity Assay), Jurkat cells transfected with the NFATLUC plasmid (containing three copies of the NFAT binding sequence operatively linked to the luciferase structural coding sequence, see above) were activated in the presence of each of the compounds that did not affect luciferase expression in the previous Toxicity Assay. The cells were activated in the presence of each compound (approximately 20 μM) as described for the Activation Assay above. Eight such compounds that did not affect the Toxicity Assay were

found to inhibit luciferase expression under the regulation of the NFAT binding sequences (Figure 9). This indicated that the inhibition of luciferase exhibited by these eight compounds in the Activation Assay (first step) was at least partly linked to NFAT-mediated transcriptional control, and not, for example, exclusively to other transcription factors such as NFκB.

Thus, of the approximately 8,000 compounds screened from this library, eight (8) compounds having experimental designations AT-1 through AT-8 were identified as having immune inhibitory activity. These eight immune inhibitors were later determined to have the structures depicted in Figures 6A and 6B. Each of the compounds in Figures 6A and 6B is also assigned a number beginning with "AQ" which can be used to purchase the compound from ArQule, Inc. (Medford, MA). The structures depicted in Figures 6A and 6B are intended to cover all isomeric forms implied by the structures as drawn.

Acute Toxicity Assay

As an additional test of the toxicity of the eight immune inhibitors identified from the primary screen above, the compounds were tested in a standard *in vivo* acute toxicity assay. Each compound (100 μg in PBS/DMSO (75%/25%)) was injected intraperitoneally into a group of two mice (one male and one female). All of the mice that received a compound survived for the 9 day observation period, after which the mice were sacrificed. Necropsies were performed and no pathology observed. Furthermore, splenocytes from mice from four groups (those that received AT-6, AT-7, AT-5, and AT-3) were tested and found to show reduced proliferation to anti-CD3 antibody *ex vivo*, as expected if T cell activation is inhibited.

Secondary Assays of Compounds Identified in the Primary Screen

Ca^{2+} Ionophore/PMA Activation Assay

The eight immune inhibitors identified in the primary screen were further assayed to determine whether the target of each immune inhibitor is likely to be located upstream or downstream of the PKC and calcineurin-dependent steps of the signaling cascade in T cells. This assay was run in basically the same manner as the Specificity Assay with cell transfected with the NFATLUC plasmid, except that cells were activated with the Ca^{2+} ionophore ionomycin (2 μM in DMSO, Calbiochem, San Diego, CA) and PMA (50 nM in ethanol, Calbiochem) for 5 - 10 minutes at room temperature (22°C). All eight immune inhibitor compounds from the primary screen inhibited luciferase expression in this assay (see Figure

10) indicating that these immune inhibitors most likely act at a target(s) downstream of calcineurin in the signaling cascade (see Figures 1 and 2).

Gel-Shift Assay (EMSA)

Gel shift assays were performed essentially as described in *Current Protocols in Molecular Biology* (Section 12.2) and Flanagan et al. (*Nature, 352*: 803 - 807 (1991)), except that DNA oligonucleotides containing the relevant transcription factor binding sequence were used. Nuclear extracts were prepared from HUT 78 cells (a transformed T cell line expressing a γδ TCR). HUT 78 cells were activated under various conditions: with PMA (32 nM, Calbiochem) and ionomycin (1 μM, Calbiochem); with PMA, ionomycin and cyclosporin A (200 ng/ml); and with PMA, ionomycin and a compound (25 μg/ml of AT-7 or AT-2) identified in the primary screen. Non-activated T cells were also used as a control. The cells were incubated for a total of two hours to allow activation by PMA and ionomycin, where present. Controls included non-activated cells, cells activated without compound, and cells activated with a validation control such as cyclosporin A which inhibits luciferase expression in cells transfected with the IL-2LUC and NFATLUC plasmids. Cells were harvested from culture medium, washed with 1X PBS, and then lysed with a low-concentration detergent buffer (for example, NP-40 at 0.2%) containing a protease inhibitor such as phenylmethylsulfonyl fluoride (PMSF). In order to prepare nuclei, the detergent must be of the right type and at the correct concentration to lyse the cell membrane but not the nuclear membrane (for example, NP-40 at 0.2%).

Nuclei in the cell lysates were then pelleted by low speed centrifugation, and the supernatant cytoplasm removed. The nuclei were then lysed in a high salt concentration (for example, 60 mM ammonium sulfate or 400 mM NaCl) buffer. Nuclear DNA-binding proteins (including nuclear transcription factors for IL-2 expression) were either collected in the supernatant of the nuclear debris pellet or were precipitated with an ammonium sulfate cut (0.3 M). Protein recoveries were quantitated by ultraviolet light absorbance (280 nm) or standard Bradford assay.

Four different oligonucleotides were synthesized by and purchased from Operon Technologies, Inc. (Alameda, CA) for use in this assay to test the ability of a compound identified in the primary screen for its ability to inhibit the binding of a particular transcription control factor to its cognate DNA sequence. The IL2 NFAT oligonucleotide was formed by annealing a DNA strand having the sequence of SEQ ID NO:3 and a

complementary DNA strand having the sequence of SEQ ID NO:4, and used to test the ability of compounds identified in the primary screen to inhibit NFAT binding to its site in the IL-2 promoter/enhancer region. The GMCSFNFAT oligonucleotide was formed by annealing a DNA strand having the sequence of SEQ ID NO:5 and a complementary sequence of SEQ ID NO:6, and used to test the ability of compounds identified in the primary screen to inhibit a NFAT binding to a different NFAT binding site found in the enhancer region of the GMCSF gene. The AP-1 oligonucleotide was formed by annealing a DNA strand having the sequence of SEQ ID NO:7 with a complementary DNA strand having the sequence of SEQ ID NO:8 and used to test the ability of a compound identified in the primary screen to inhibit AP-1 binding to this site from the IL-2 promoter/enhancer region. The NFκB oligonucleotide was formed by annealing a DNA strand having the sequence of SEQ ID NO:9 with a complementary DNA strand having the sequence of SEQ ID NO:10 and used to test the ability of a compound identified in the primary screen to inhibit NFκB binding to its cognate DNA sequence in the IL-2 promoter/enhancer region.

Binding reactions were generally performed with 2 ng of ^{32}P end-labeled DNA fragments or oligonucleotides containing relevant transcription factor binding sites. Reaction volumes were typically around 10 to 20 µl in binding buffer (10 mM HEPES (pH 7.5), 100 mM NaCl, 20% glycerol, 1 mM DTT, and non-specific DNA such as poly-dI/dC at 200 ng per reaction). Typically, 2 - 5 µg of nuclear DNA-binding proteins (see above) were incubated with the labeled DNA in binding buffer for 15 minutes at room temperature. Bound and unbound DNA were then separated by electrophoresing on non-denaturing poly-acrylamide gels (6% acrylamide in 1X TBE buffer (GIBCO BRL)). Gels were dried and exposed to X-ray film.

Nuclei were extracted with ammonium sulfate, and crude nuclear protein extracts were prepared for gel shift assays as described above. Normalized amounts of extracts were incubated with labeled DNA fragments for 15 minutes at room temperature.

Binding reactions were loaded onto 6% acrylamide gels in 1X TBE and were run at 120 volts at room temperature. The gels were dried on a vacuum drier and exposed to X-ray film. Activation in controls caused the induction of binding activities specific for both the NFAT and AP-1 sites resulting in slower mobility bands in the lanes of the gels containing DNA and nuclear proteins from activated cell extracts than in lanes of the gels containing DNA and nuclear proteins from non-activated cell or cells activated in the presence of

cyclosporin A. Cyclosporin A blocked the formation of NFAT/DNA and AP-1/DNA complexes as expected.

Two (AT-7 and AT-2) of the eight compounds identified as immune inhibitors in the primary screen were assayed and found to block the formation of the NFAT/DNA complex formation but actually to enhance the formation of the AP-1/DNA complex, indicating that these compounds work via a different mechanism than cyclosporin A.

Three of the eight compounds (AT-2, AT-7, and AT-8) identified in the primary screen as immune inhibitors were assayed again using an improved gel-shift assay protocol. The improved gel shift assays were run as described with the following changes. Nuclei were prepared from Jurkat cells as used in the primary screen. The nuclei were lysed using a high salt concentration (400 nM NaCl) buffer. The nuclear DNA-binding protein complexes (including nuclear transcription factors for IL-2 expression) were collected in the supernatants of the nuclear debris pellet. Reaction volumes were 20 µl in binding buffer (20 mM HEPES (pH 7.5), 35 mM NaCl, 35 nM KCl, 15% glycerol, 1 mM EDTA, 1 mM DTT, 100 µg/ml BSA, and non-specific DNA such as poly-dI/dC at 1 µg per reaction). Typically, 5 - 7.5 µg of the nuclear DNA-binding protein complexes were incubated with the labeled DNA in binding buffer for 15 minutes at room temperature. Bound and unbound DNA were separated by electrophoresis with 120 V at room temperature on non-denaturing poly-acrylamide gels (4% or 5% acrylamide in 0.5X TBE buffer (GIBCO BRL) for IL2NFAT and GMCSFNFAT or AP-1 and NFκB, respectively). In this improved assay, Cyclosporin A blocked the formation of NFAT/DNA complex and reduced the number of AP-1/DNA complexes as expected. In addition, using this improved gel shift assay protocol, compounds AT-2 and AT-8 were found to block the formation of the NFAT/DNA complex, strongly inhibited the NFκB/DNA interaction and reduced the amount of AP-1/DNA complexes formed. The immune inhibitor compound AT-7 had only minor effects on any of these three complexes, indicating that AT-7 has a different mode of action than AT-2 and AT-8.

NFATp Dephosphorylation

Approximately 10 to 20 x 10^6 HUT78 cells were incubated for 15 minutes at room temperature (22°C) in the absence or presence a compound (25 µg/ml) identified as an immune inhibitor in the primary screen or, as a positive control, in the presence of cyclosporin A (Sigma Chemical Co., St. Louis, MO) or FK506 (100 ng/ml). The cells were then activated in solution either with 10 µg/ml of the anti-TCR MAb 16G8 (Endogen) and 20

μg/ml of goat anti-mouse antibody or with 2 μM ionomycin (Calbiochem) in DMSO and 50 nM phorbol-12-myristate-13-acetate (PMA, Calbiochem) in ethanol for 5 to 10 minutes at room temperature (22°C). The cells were washed in ice-cold phosphate buffered saline (PBS) and lysed in RIPA buffer (20 mM Tris[hydroxymethyl] aminomethane, pH 7.6; 300 mM NaCl; 5 mM EDTA ([ethylenedinitrilo] tetraacetic acid); 5 mM, EGTS (ethylene glycol-bis-[β-aminoethyl ether]-N, N, N', N'-tetraacetic acid); 1 % (v/v) Triton X-100; 1 % (v/v) deoxycholate; 0.1 % (w/v) sodium dodecyl sulfate (SDS); 50 mM pyrophosphate, 50 mM NaF; 20 μM $ZnCl_2$; 500 μM orthovanadate; 1 mM phenylmethylsulfonyl fluoride (PMSF); 10 μg/ml aprotinin; 10 μg/ml leupeptin; 50 μg/ml pepstatin; 50 μg/ml trypsin inhibitor from soybean). NFATp was immunoprecipitated overnight from 2.5 mg of lysate protein with 5 μl of anti-NFATp-antibody (#06-348, Upstate Biotechnology, Inc., Lake Placid, NY) at 4°C.

The immunoprecipitates were washed with RIPA buffer, boiled in SDS-PAGE sample buffer (50 mM Tris, pH 6.8, 2% (v/v) 2-mercaptoethanol, 2% (w/v) SDS, 10% glycerol, bromphenol blue), and electrophoresed on SDS-polyacrylamide gels. The proteins were transferred from the gels to nitrocellulose membranes for detection of NFATp species by immunoblotting with anti-NFATp antibody (Western blot analysis) and horseradish peroxidase-protein A (Kirkegaard and Perry, Gaithersburg, MD).

Western blot analysis of immunoprecipitates from HUT78 cells with anti-NFATp antibody detected two forms of NFATp. In non-activated cells or cells activated in the presence of either cyclosporin A or FK506 (50 ng/ml), NFATp exhibited an apparent Mr of 130,000 daltons on SDS-gels which represented the phosphorylated form of NFATp. Activation of T cells with either the anti-T cell antigen receptor 16G8 MAb or ionomycin plus PMA led to dephosphorylation of NFAT. This dephosphorylated form ran on SDS-gels with an apparent Mr of approximately 120,000 daltons. Activation of T cells in the presence of 25 μg/ml of any of the eight compounds identified as immune inhibitors in the primary screen did not prevent the dephosphorylation of NFATp. This result indicates that none of the compounds inhibited the activity of the phosphatase calcineurin which is responsible for the dephosphorylation of NFATp. Accordingly, unlike cyclosporin A, the immune inhibitors identified in the primary screen must act at a target molecule other than calcineurin in the signaling cascade.

Raf, MEK, and ERK Activity Assays

Initially, compounds identified in the primary screen as immune regulators were assayed for ERK Activity using the following protocol. Cells were activated with either the anti-TCR MAb 16G8 and goat anti-mouse antibody (16G8 anti-mouse antibody was used with Jurkat; Ionomycin/PMA was used for Jurkat and HUT78) or with ionomycin and PMA as described for the NFAT dephosphorylation assay. Cells were lysed using RIPA buffer (see above) or lysis buffer (50 mM HEPES (4-[2-hydroxethyl]-1-piperazineethanesulfonic acid) (pH 7.5), 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM pyrophosphate, 50 mM NaF, 20 μM $ZnCl_2$, 500 μM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml pepstatin, 50 μg/ml trypsin inhibitor from soybean). ERK activation was assessed by immunoblotting of 10 µg/ml of lysate protein with an anti-ERK2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or by immune complex kinase assay. For the *in vitro* kinase assay, ERK2 was immunoprecipitated with 10 µl anti-ERK2 antibody from 500 µg of lysate protein as described above. After washes with lysis buffer, the immune complex was washed with 1 ml of preK-buffer (25 mM HEPES (pH7.5), 5 mM NaF, 5 μM $ZnCl_2$, 20 mM β-glycerophosphate, 10 mM pNPP (p-nitrophenyl phosphate), 10 mM $MgCl_2$, 2 mM DTT (Dithiothreitol), 200 μM orthovanadate). The kinase reaction was performed for 20 minutes at room temperature (22°C) in kinase-buffer (preK-buffer plus 20 μM ATP, 10 μCi of [γ-^{32}P] ATP (NEN-Dupont)) in the presence of 2 μg MBP (myelin basic protein, Sigma Chemical Co., St. Louis, MO) after which it was stopped by boiling in sample buffer. After separation on a SDS-gel, the dried gel was exposed to film.

This analysis showed that ERK2 in lysates from non-activated cells ran as one band with a Mr of approximately 42,000 daltons. However, upon activation of cells, an additional band of approximately 44,000 daltons appeared in the lysates. This represented the activated, threonine- and tyrosine-phosphorylated form of ERK2. Activation in the presence of compounds identified as immune inhibitors in the primary screen did not decrease the amount of shifted ERK2, suggesting that none of the compounds inhibited the crucial Ras pathway.

The compounds AT-2, AT-7, and AT-8 were assayed again for Raf, MEK, and ERK activity using the above protocol with the following modifications. Cells were activated with either anti-TCR MAb 16G8 (cross-linked with goat anti-mouse antibody), with ionomycin and PMA as described for the NFAT dephosphorylation assay or with 50 ng/ml TNFα

(rhuTNFα, #4130-60, Intergen, Purchase, NY). Raf, MEK, ERK activation was assessed by immunoblotting of 50, 50 or 10 μg lysate with anti-Raf-1, anti-MEK1 or anti-ERK2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), respectively, or by immune complex kinase assay. For the *in vitro* kinase assay, Raf-1, MEK1 and ERK2 were immunoprecipitated with 25, 10 or 5 μl antibody, respectively, from 1 mg of lysate protein as described above. The kinase reaction was performed for 20 minutes at room temperature (22°C) in kinase-buffer (preK-buffer plus 20 μM ATP, 10 μCi of [γ-^{32}P] ATP (NEN-Dupont)) in the presence of 3 μg His-MEK, 5 μg His-ERK or 5 μg MBP as substrates for Raf-1, MEK1 or ERK2, respectively, after which it was stopped by boiling in sample buffer. This analysis showed that none of the three compounds tested, AT-2, AT-7 and AT-8, inhibited Raf-1, MEK1 or EKR2-kinase activity irrespective of cell type or stimulation. These results were confirmed by the use of the simpler western blot assay. Taken together, the data suggest that none of the compounds inhibits the crucial Ras pathway.

JNK Activity Assay

An *in vitro* kinase assay was used as a quantitative measurement of JNK activation as activated JNKs, in contrast to ERKs, do not appear to significantly shift in SDS-gels. For the JNK assay, JNK1 was immunoprecipitated with 10 μl antibody from 1 - 1.5 mg of lysates obtained from cells stimulated in the absence or presences of the immune inhibitors AT-2, AT-7 or AT-8 (30 μg/ml). Subsequent steps were performed as described in the ERK activity assay (see, above). However, 10 μg of GST-c-jun-1-79 were used as a substrate. JNK1 kinase activity from unstimulated cells showed a low background phosphorylation of GST-c-jun which was enhanced 10x by activation via ionomycin/PMA. In the presence of AT-2 the kinase activity was inhibited by 50% whereas AT-7 and AT-8 did not show any significant effect. Some of the proteins directly upstream of JNK are shared by different signaling pathways which are stimulated by anti-TCR or TNFα, respectively (see Figure 1, MEKK1 and MKK4). There is some evidence that the crosstalk between the Ras- and the JNK-pathway takes place at MEKK1 (Russell et al., *J. Biol. Chem., 270*: 11575 - 11760 (1995)). Therefore, JNK1 kinase assays were performed on lysates from cells stimulated with TNFα (rhuTNFα, #4130-60, Intergen, Purchase, NY). JNK1 kinase activity from cells stimulated with TNFα in the presence of 30 μg/ml of AT-2 was not inhibited. Compounds AT-7 or AT-8 also did not show any effect. Qualitatively similar results were obtained when Jurkat or U937 (monocytes) cells were used. The data suggest that AT-2 does not directly act on JNK1

but rather interferes with the crosstalk from the Ras to the JNK pathway.

Nuclear Translocation

Approximately 100 x 10^6 T cells were pre-incubated for 15 minutes at room temperature in the absence or presence of 30 μg/ml of the immune regulator AT-7 from the primary screen, then activated either with 10 μg/ml anti-T cell receptor antibody (16G8, Endogen, Cambridge, MA) and 20μg/ml goat-anti-mouse antibody or with 2 μM ionomycin in DMSO (Calbiochem) and 50 nM PMA (Calbiochem) in ethanol for 5 to 10 minutes at room temperature. After a wash with ice-cold PBS (GIBCO BRL), the cells were lysed in Cytb buffer (10 mM HEPES (pH 7.3), 15 mM KCl, 2.5 mM $MgCl_2$, 0.25% NP-40, 0.1 mM EDTA, 20 μM $ZnCl_2$, 25 mM NaF, 10 mM pyrophosphate, 500 μM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml pepstatin, 50 μg/ml trypsin inhibitor from soybean). The lysed cells were centrifuged at 5,000 rpm for 1 minute. The supernatant was the "cytosolic extract". The pellet was washed with Cytb buffer without NP40 and then extracted with 420-buffer (Cytb buffer plus 10% glycerol and 420 mM NaCl) for 30 minutes with rocking at 4°C. The extract was centrifuged (for example, for 12 minutes. At 14,000 rpm in an Eppendorf tabletop centrifuge). The supernatant was the "nuclear extract".

NFATp was immunoprecipitated with anti-NFATp-antibodies from cytosolic and nuclear extracts of non-activated (control) and activated cells in the absence and presence of immune inhibitor. Western blot with anti-NFATp-antibodies showed that NFATp in activated cells was dephosphorylated and ran faster on SDS-polyacrylamide gels. In addition, in non-activated (control) cells, phosphorylated NFATp remained in the cytosolic fraction whereas the dephosphorylated protein translocated into the nucleus (i.e., seen in lanes loaded with nuclear extract).

A preliminary result of this assay suggested that NFAT dephosphorylation and translocation to the nucleus was not inhibited by compound AT-7 at 30 µg/ml.

Splenocyte Proliferation Assay

The ability of the immune inhibitors identified in the primary screen to inhibit proliferation of non-transformed cells was assessed. The compounds were incubated with splenocytes from naive DBA-2 mice. In particular, splenocytes were cultured in 96-well plates in the identical medium described above for growing Jurkat cells (RPMI 1640 with 10% fetal bovine serum) at 1 X 10^6 cells per well in the presence of the poly-clonal T cell

stimulator Concanavilin A (ConA, Calbiochem, San Diego, CA) at 0.3, 1 or 3 μg/ml. Cells were incubated in the presence of the eight compounds (25 μg/ml) for 72 hours. The cultures were then pulse-labeled with 1 μCi/well of ^{3}H-thymidine (Dupont NEN, Boston, MA) for another 24 hours and harvested onto glass fiber filters for scintillation counting (Packard, Meriden, CT).

All eight compounds inhibited proliferation to some extent (Figure 11). Generally, the highest level of inhibition by the compounds was observed at the lowest concentration of ConA. Compound AT-2, however, inhibited proliferation at all three ConA concentrations to at least 100%.

Balb-c mice injected with AT-2 or cyclosporin A mixed with the Sperm Whale Myoglobin (SWM) peptide (SEQ ID NO: 11 showing amino acids 110 to 121 of SWM, synthesized by and purchased from QCB, Hopkinton, MA; see also, Infante et al., *J. Exp. Med., 154*: 1342 -1356 (1981)) in complete Freund's adjuvant (CFA, Sigma Chemical Co.) emulsion were sacrificed on day 21 and splenocytes were cultured with either antigen (SWM peptide) or with anti-CD3 antibody (2C11, Pharmingen, San Diego, CA). Cells were cultured as above except that the medium was HL-1 (Biowhitaker, Walkersville, MD) supplemented with glutamine, penicillin, streptomycin, and 2-mercaptoethanol (GIBCO BRL, Gaithersburg, MD amounts as above) and with sodium pyruvate and non-essential amino acids (both 100 μM, GIBCO BRL). Antigen-specific T cells were stimulated with SWM peptide between 350 and 0.3 μM for 72 hours. Cultures were then pulse-labeled and counted as above. Control cultures were set up with the 2C11 antibody (2.5 μg/ml to 0.1 ng/ml) instead of SWM peptide. The results were plotted as stimulation indices (CPM of thymidine incorporation with antigen divided by CPM without antigen). The results show that T cells from mice treated with the highest dose of AT-2 did not proliferate in response to the SWM antigen (see Figure 12), similar to unprimed mice (negative control). By comparison, cyclosporin A-treated mice responded similarly to primed mice without either cyclosporin A or AT-2 (positive control) since cyclosporin A does not induce long-term tolerance. Control cultures with the anti-CD3 antibody 2C11 show no striking differences between groups.

Delayed-Type Hypersensitivity (DTH) Assay Using AT-8

The immune inhibitor AT-8 identified in the primary screen above was also tested in a standard Delay-Typed Hypersensitivity (DTH) Assay as an indication of the compound's ability to suppress T cell activation. On Day 0, mice were injected subcutaneously at the base

of the tail with Sperm Whale Myoglobin (SWM) peptide in complete Freund's adjuvant (CFA) in the presence of 10, 1, 0.1, or 0 mg of AT-8. An unprimed control received CFA alone without SWM peptide. A cyclosporin A control received 2 mg of cyclosporin A. On Day 6, each mouse received a subcutaneous injection in one rear footpad of SWM peptide (50 µg per mouse) in 25 µl PBS. The other rear footpad (uninjected) of the same mouse served as the control. On Day 7, the footpads were examined for edema in the injected foot as compared to the control footpad. The results indicate that CsA (2 mg) and AT-8 at 1 and 10 mg significantly suppressed edema due to antigen-specific T cell activation ($p<0.05$) compared to controls (Figure 13).

A summary of the characterization of the immune inhibitors identified in the Primary Screen and Secondary Assays is presented below:

	CsA	AT -2	AT -7	AT -8	AT -6	AT -5	AT -1	AT -3	AT -4
IL2-Luc [αTCR/P]	-	-	-	-	-	-	-	-	-
IL2-Luc [I/P]	-	-	-	-	-	-	-	-	-
β-actin-Luc	0	0	0	0	0	0	0	0	0
NFAT-Luc [αTCR/P]	-	-	-	-	-	-	-	-	-
NFAT-Luc [I/P]	-	-	-	-	-	-	-	-	-
AP-1-Luc [P]	0	-	-	-	-				
Splenocyte prol.	-	--	-	--	-	-	-	--	-
IL2-NFAT-oligo	no	no	less	no					
GMCSF-NFAT-oligo	no	no	less	no					
AP1-oligo	yes	less	yes	less					
NFκB-oligo	yes	no	yes	no					
NFAT-dephosph.	no	yes	yes	yes	yes	yes	yes	yes	yes
ERK2 shift [I/P-Hut]	yes	yes	yes	yes+	yes+	yes+	yes+	yes+	yes+
ERK2 shift [αTCR-J]	yes	(yes)	(yes)	(yes)	(yes)	(yes)	(yes)	(yes)	(yes)
ERK2 shift [I/P-J]	yes	yes	yes	yes					
Raf-1 shift [I/P-J]	yes	yes	yes	yes					
ERK2 activity [I/P]	0	0	0	0					
MEK1 activity [I/P]			0	0					
Raf-1 activity [I/P]			0	0					

JNK1 activity [TNFα]	0	0	0	0
JNK1 activity [I/P]	-	-	0	0
nucl.transl. [NFATp]	no		yes	

"-" = inhibition / less, "+" = activation / more, 0 = no effect, αTCR/P = anti-TCR antibody and PMA, I/P = ionomycin and PMA, J = Jurkat cells, Hut = HUT78 cells, AT-1 is AQ32778, AT-2 is AQ32705, AT-3 is AQ32785, AT-4 is AQ32809, AT-5 is AQ32687, AT-6 is AQ29268, AT-7 is AQ25958, and AT-8 is AQ588 (ArQule, Inc., Medford, MA).

Example 2. Immune Activators Identified in the Primary Screen

The primary screen (Activation Assay) identified 18 compounds which activated luciferase expression (i.e., greater than 125% activation relative to control (no compound) 100%, see Figure 14). Of those eighteen, eight (designated A, B, C, D, K, M, S, and Y in Figures 15 and 16) were determined in the Toxicity Assay to not be general transcription activators and, therefore, were next examined in the third step of the primary screen (Specificity Assay). The Toxicity Assay was able to identified a particularly potent general transcription activator (O in Figure 15) which was not studied further. All eight compounds also activated luciferase expression in the Specificity Assay using cells transfected with the NFATLUC plasmids (see Figure 17).

Example 3. Oxazolone Sub-library

Based on the results of screening the library of approximately 8,000 synthesized oxazalone-derivative arylidene diamide compounds described in Examples 1 and 2, a sub-library was made consisting of 79 additional oxazolone-derivative arylidene diamide compounds that were synthesized based on the structure of compound AT-2 (see Figure 6A). Of the 79 derivative compounds, 73 were identified as having a positive result in the first step (Activation Assay) of the primary screen. These 73 compounds are being further screened and characterized as in Examples 1 and 2 above.

Example 4: Screening a Library of 3,840 Organic Compounds

Another library consisting of 3,840 synthetic organic compounds synthesized by combinatorial chemistry (Hogan, *Nature, 384 (Supp.)*: 17 - 19 (1996)) was screened using the primary screen and secondary assays as described in Examples 1 and 2. The Activation Assay of the primary screen identified 62 compounds as having a potential immune

regulatory activity. 11 of these 62 were eliminated by the Toxicity Assay and 42 of the remaining compounds had some specific effect on NFAT-mediated gene expression (Specificity Assay). These compounds were further characterized by secondary assays as described above.

The Activation Assay of the primary screen was also used to calculate the concentration at which 50 per cent (IC_{50}) and 90 per cent (IC_{90}) of the cells in the assay were inhibited from activation by compounds M3.5H8 and M4.6H5. These two compounds were then further assayed using flow cytometry for the ability to induce apoptosis. A standard apoptotic assay of this example uses flow cytometry to detect the staining of cells with one or two molecules which emit a different fluorescent signal that can be detected simultaneously by a flow cytometer to determine whether cells are undergoing early apoptosis, are viable, or are necrotic. Cells undergoing early apoptosis will be bound on their surface (stained) by fluorescein isothiocyanate (FITC)-labeled Annexin V, but will exclude the DNA intercalating fluorescent dye propidium iodide (PI). Viable cells not undergoing apoptosis will not be stained significantly by FITC-labeled Annexin V and will not be stained by PI. Necrotic cells stain positive with both FITC-labeled Annexin V and PI.

Approximately 2 x 10^5 Jurkat cells (1 x 10^6 cells/ml) were incubated in the presence of increasing concentrations of each test compound from the primary screen for 1 to 24 hours at 37°C. The cells were pelleted in a microcentrifuge (1000 rpm, 5 minutes, room temperature), washed once with 3 ml of phosphate buffered saline (PBS)/bovine serum albumin (BSA, 1 % w/v), pelleted again, and resuspended into 100 µl of 1X binding buffer (Kamiya Biomedical Company, Seattle, WA). Cells were mixed with FITC-labeled-Annexin V (Kamiya Biomedical Company, Seattle, WA) alone, at a final concentration of 400 ng/ml, or were mixed simultaneously with both FITC-Annexin V (400 ng/ml) and propidium iodide (PI, 5 µg/ml), followed by incubation in the dark for 10 to 20 minutes at room temperature. The cells were then removed from the dark and washed with 3 ml of PBS/1 % BSA, pelleted as described above, and resuspended into 500 µl of PBS/1 % BSA, and analyzed using a flow cytometer (FACSCAN™, Becton & Dickinson, San Jose, CA) equipped with a single laser emitting excitation light at 488 nm. Cells were incubated in the presence or absence of M3.5H8 and M4.6H5 for 4 hours at 37°C. The results are shown in Figures 18A - 18F. Figure 18A shows the results of incubating cells in the absence of compound (control). Figure 18B shows the results of incubating cells in the presence of antibody to Fas (Kamiya

Biomedical Company, Seattle, WA) available from which induces apoptosis as indicated as detection of fluorescently labeled cells in the M2 region of the graph (positive control). Figures 18C and 18D show the results of incubating cells in the presence of M3.5H8 at the IC_{50} and IC_{90} concentrations of 60 ng/ml and 2 μg/ml, respectively. Figures 18E and 18F show the results of incubating cells in the presence of M4.6H5 at the IC_{50} and IC_{90} concentrations of 60 ng/ml and 5 μg/ml, respectively. The results show that neither of the two immune inhibitor compounds induced apoptosis in the assay.

Example 5: Screening a Library of 17,920 Synthetic Compounds

Another library consisting of 17,920 synthesized compounds was run through the primary screen as basically as described in Example 1 above. To reduce the amount of manual manipulation and time needed to read samples from the primary screen, it is desirable to use a luciferase substrate formulation which provides a light signal which is stable and has a half-life as long as possible. Such an improved luciferase formulation is LUC-LIGHT™ (Packard, Meriden, CT) which provides a light signal with a half-life of greater than 5 hours. This formulation permitted the use of stacking devices which sequentially feed a series of plates into the luminometer.

The Activation Assay of the primary screen identified 323 compounds as having a potential immune inhibitory activity. Of these 323 compounds, 94 were eliminated by the Toxicity Assay as having a non-specific effect on transcription. The Specificity Assay using NFATLUC as in Example 1, identified 130 compounds as having a specific effect on NFAT-mediated gene expression. These compounds are being further characterized by secondary assays as described above.

In addition, 323 compounds of the library were identified in the primary screen performed with an 18 hour incubation period as immune stimulatory compounds. These compounds were further screened for anti-apoptotic activity. Jurkat cells transfected with β-actinLUC were incubated for 18 hours in the presence or absence of PMA, anti-TCR and PMA, or anti-fas antibody with or without cyclosporin A. Cyclosporin A is known to inhibit T cell antigen receptor induced expression of fasL (Dhein et al., *Nature, 377*: 438 - 441 (1995)) and serves as a positive control. Figure 20 shows expression of luciferase (light units) versus stimulation conditions versus concentration of cyclosporin A (CsA, ng/ml) in cells in the modified Toxicity Assay transfected with β-actinLUC and incubated for 18 hours

under the indicated conditions. One of these compounds was identified as an anti-apoptotic agent as demonstrated by activation of reporter gene expression in activated, but not in unactivated, cells transfected with β-actinLUC (see Figure 21).

Example 6: Screening a Library of 216 Fungal Extract Compositions

Another library of 216 fungal culture extract compositions prepared from 61 different fungal strains and 6 different media and growth conditions were screened using the primary screen described above. Nine of these compositions exhibited inhibitory activity in the first step (Activation Assay) of the primary screen using the IL2-LUC construct. Two of these nine compositions were eliminated in the second step (Toxicity Assay) using the β-actinLUC construct. The remaining seven fungal extract compositions exhibited inhibitory activity in the third step (Specificity Assay) using the NFATLUC construct as described in Example 1. Two of the seven fungal extracts that inhibited the NFATLUC showed particularly potent immune inhibitory in the Specificity Assay of the primary screen. These seven compositions prepared from fungal cultures are being further characterized using various secondary assays as described above.

Example 7: Screening a Library of 1020 Fungal Extract Compositions

Another library of 1020 compositions were prepared from fungal cultures and screened by the primary screen as described in Example 1 above. 29 of these compositions showed a potential immune inhibitory activity by the Activation Assay (IL-2LUC construct) of the primary screened. 14 of these 29 were eliminated by the Toxicity Assay using Jurkat cells transfected with the β-actinLUC construct. 12 of the remaining 14 were identified by the Specificity Assay as regulating NFAT-mediated gene expression. These 12 fungal extract compositions from the primary screen are being further characterized by secondary assays as described above. One other composition, XG08, showed strong immune stimulatory activity in the primary screen. This composition augmented IL-2 production from anti-CD3 stimulated splenocytes (see Figure 19).

All of the publications and copending U.S. applications specifically referred to above are hereby incorporated by reference.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: T Cell Sciences, Inc.

(ii) TITLE OF INVENTION: Method of Isolating Regulators of T Cell Activation

(iii) NUMBER OF SEQUENCES: 11

(iv) CORRESPONDENCE ADDRESS:

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: WordPerfect 6.1

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: not yet assigned

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(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Thomas R. Berka, Ph.D.

(B) REGISTRATION NUMBER: 39,606

(C) REFERENCE/DOCKET NUMBER: 412.1 PCT (11600.04323)

(ix) TELECOMMUNICATION INFORMATION:

(B)TELEFAX:617-345-9111

(2) INFORMATION FOR SEQ ID NO:1:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH:75 base pairs

(B)TYPE:nucleic acid

(C)STRANDEDNESS:single

(D)TOPOLOGY:linear

(ii)MOLECULE TYPE:DNA

(xi)SEQUENCE DESCRIPTION:SEQ ID NO:1:

TCGAGAATTC CAAAGAGTCA TCAGAAGAAA TTCCAAAGAG TCATCAGAAG 50

AAATTCCAAA GAGTCATCAG AAGAC 75

(2) INFORMATION FOR SEQ ID NO:2:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH:75 base pairs

(B)TYPE:nucleic acid

(C)STRANDEDNESS:single

(D)TOPOLOGY:linear

(ii)MOLECULE TYPE:DNA

(xi)SEQUENCE DESCRIPTION:SEQ ID NO:2:

TCGAGTCTTC TGATGACTCT TTGGAATTTC TTCTGATGAC TCTTTGGAAT 50

TTCTTCTGAT GACTCTTTGG AATTC 75

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:35 base pairs

(B) TYPE:nucleic acid

(C) STRANDEDNESS:single

(D) TOPOLOGY:linear

(ii) MOLECULE TYPE:DNA

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:3:

TCGAGGAAAG GAGGAAAAAC TGTTTCATAC AGAAC 35

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:35 base pairs

(B) TYPE:nucleic acid

(C) STRANDEDNESS:single

(D) TOPOLOGY:linear

(ii) MOLECULE TYPE:DNA

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:4:

TCGAGTTCTG TATGAAACAG TTTTTCCTCC TTTCC 35

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:32 base pairs

(B) TYPE:nucleic acid

(C) STRANDEDNESS:single

(D) TOPOLOGY:linear

(ii) MOLECULE TYPE:DNA

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:5:

TCGAGCACAC ATCTTTCTCA TGGAAAGATG AC 32

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:32 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS:single

(D) TOPOLOGY:linear

(ii) MOLECULE TYPE:DNA

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:6:

TCGAGTCATC TTTCCATGAG AAAGATGTGT GC 32

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:29 base pairs

(B) TYPE:nucleic acid

(C) STRANDEDNESS:single

(D) TOPOLOGY:linear

(ii) MOLECULE TYPE:DNA

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:7:

TCGAGAATTC CAAAGAGTCA TCAGAAGAC 29

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:29 base pairs

(B) TYPE:nucleic acid

(C) STRANDEDNESS:single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TCGAGTCTTC TGATGACTCT TTGGAATTC 29

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TCGAGCAAAG AGGGATTTCA CCTACATCC 29

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TCGAGGATGT AGGTGAAATC CCTCTTTGC 29

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:12 amino acids

(B) TYPE:amino acid

(C) STRANDEDNESS:single

(D) TOPOLOGY:linear

(ii) MOLECULE TYPE:peptide

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:11:

Ala Ile Ile His Val Leu His Ser Arg His Pro Gly 12

We claim:

1. A method for identifying a composition or compound that regulates T cell activation, comprising the steps of:

(a) determining whether the composition or compound inhibits or stimulates expression of a reporter gene in an activated T cell transfected with a first reporter gene construct, wherein the first reporter gene construct comprises a promoter/enhancer region of a cytokine gene involved in early T cell activation operatively linked to the structural coding sequence of the reporter gene;

(b) determining whether the composition or compound inhibits or stimulates expression of a reporter gene in an activated T cell transfected with a second reporter gene construct, wherein the second reporter gene construct comprises a transcriptional control sequence of a ubiquitously expressed gene operatively linked to the structural coding sequence of the reporter gene;

(c) determining whether the composition or compound inhibits or stimulates expression of a reporter gene in an activated T cell transfected with a third reporter gene construct, wherein the third reporter gene construct comprises a plurality of copies of a sequence which is bound by a particular transcription factor for a gene involved in T cell activation and which is operatively linked to the structural coding sequence of the reporter gene; and

(d) selecting the composition or compound which stimulates or inhibits reporter gene expression in steps (a) and (c) and which does not significantly stimulate or inhibit reporter gene expression in step (b) relative to the levels of expression of the reporter gene in the T cells activated in the absence of the composition or compound in each step.

2. The method for identifying a composition or compound that regulates T cell activation according to claim 1 wherein the reporter gene is selected from the group consisting of the luciferase gene, the lac gene, and the chloramphenicol acetyltransferase gene.

3. The method according to claim 2 wherein the reporter gene is the luciferase gene.

4. The method for identifying a composition or compound that regulates T cell activation according to claim 1 wherein the promoter/enhancer region in step (a) is the IL-2

promoter/enhancer region.

5. The method for identifying a composition or compound that regulates T cell activation according to claim 1 wherein the transcriptional control sequence in step (b) is from the β-actin gene.

6. The method for identifying a composition or compound that regulates T cell activation according to claim 1 wherein the transcription factor in step (c) is selected from the group consisting of NFAT, AP-1, octamer-binding complex, and NF-κB.

7. The method according to claim 6, wherein the transcription factor in step (c) is NFAT.

8. The method for identifying a composition or compound that regulates T cell activation according to claim 1 wherein the T cells are activated by incubating the T cells with a molecule selected from the group consisting of an antibody against the T cell antigen receptor, an antibody against CD3 on the surface of a T cell, a lectin protein that binds the T cell antigen receptor, a lectin protein that binds CD3, phorbol myristate acetate, a calcium ionophore, and combinations thereof.

9. The method for identifying a composition or compound that regulates T cell activation according to claim 1 further comprising the step of determining whether a composition or compound of step (d) inhibits or stimulates expression of a reporter gene in an activated T cell transfected with a fourth reporter gene construct, wherein the fourth reporter gene construct comprises a plurality of copies of a sequence which is bound by a transcription factor other than the particular transcription factor in step (c) and which is operatively linked to the structural coding sequence of the reporter gene.

10. The method according to claim 9 further comprising performing an assay to determine whether the composition or compound inhibits or stimulates an assay selected from the group consisting of a gelshift assay using a transcription factor binding site from a gene involved in T cell activation, an NFATp dephosphorylation assay, a phosphotyrosine protein assay, a pp120 dephosphorylation assay, a nuclear cdc2 assay, a nuclear cdk2 assay, a Raf activity

assay, an MEK activity assay, an ERK activity assay, a JNK activity assay, a nuclear translocation of proteins assay, a protein kinase C assay, a fos/jun protein expression assay, T cell proliferation assay, cytokine production assay, calcium ion flux assay, an apoptosis assay, and combinations thereof.

11. A method for identifying a composition or compound that inhibits apoptosis, comprising the steps of:

(a) determining whether the composition or compound stimulates expression of a reporter gene in a T cell transfected with a first reporter gene construct, wherein the first reporter gene construct comprises a promoter/enhancer region of a cytokine gene involved in early T cell activation operatively linked to the structural coding sequence of the reporter gene under conditions where the T cell is incubated with the composition or compound for greater than 8 hours;

(b) determining whether the composition or compound stimulates expression of a reporter gene in both activated and unactivated T cells transfected with a second reporter gene construct, wherein the second reporter gene construct comprises a transcriptional control sequence of a ubiquitously expressed gene operatively linked to the structural coding sequence of the reporter gene, and wherein the activated and unactivated T cells are incubated in the presence of the composition or compound for greater than 8 hours; and

(c) selecting the composition or compound which stimulates reporter gene expression in step (a) and which stimulates reporter gene expression in the activated T cells but not the unactivated T cells in step (b).

12. The method of identifying a composition or compound that inhibits apoptosis according to claim 11, wherein the reporter gene is selected from the group consisting of the luciferase gene, the lac gene, and the chloramphenicol acetyltransferase gene.

13. The method of identifying a composition or compound according to claim 12 wherein the reporter gene is the luciferase gene.

14. The method for identifying a composition or compound that inhibits apoptosis according to claim 11 wherein the promoter/enhancer region in step (a) is the IL-2

promoter/enhancer region.

15. The method for identifying a composition or compound that inhibits apoptosis according to claim 11 wherein the transcriptional control sequence in step (b) is from the β-actin gene.

16. The method for identifying a composition or compound that inhibits apoptosis according to claim 11, further comprising the step of performing an apoptosis assay using flow cytometry to detect inhibition of translocation of phosphatidylserine from the cytosolic side to the extracellular side of the plasma membrane of a cell or an apoptosis assay using gel electrophoresis to detect inhibition of apoptosis-dependent fragmentation of nucleosomal DNA.

17. The method for identifying a composition or compound that inhibits apoptosis according to claim 11, wherein the T cells in step (b) are induced to undergo apoptosis by crosslinking the T cell antigen receptor or fas on the surface of the T cells.

18. A composition or compound that regulates T cell activation identified according to the method in claim 1.

19. A composition or compound that regulates T cell activation identified according to the method in claim 9.

20. A compound identified as an immune regulator by the method according to claim 1 selected from the group consisting of AT-1, AT-2, AT-3, AT-4, AT-5, AT-6, AT-7, AT-8 in Figures 6A and 6B.

21. The compound according to claim 20 wherein said compound inhibits NFAT-mediated transcription of genes without inhibiting calcineurin activity.

22. A composition or compound that inhibits apoptosis identified by the method according to claim 11, 16, or 17.

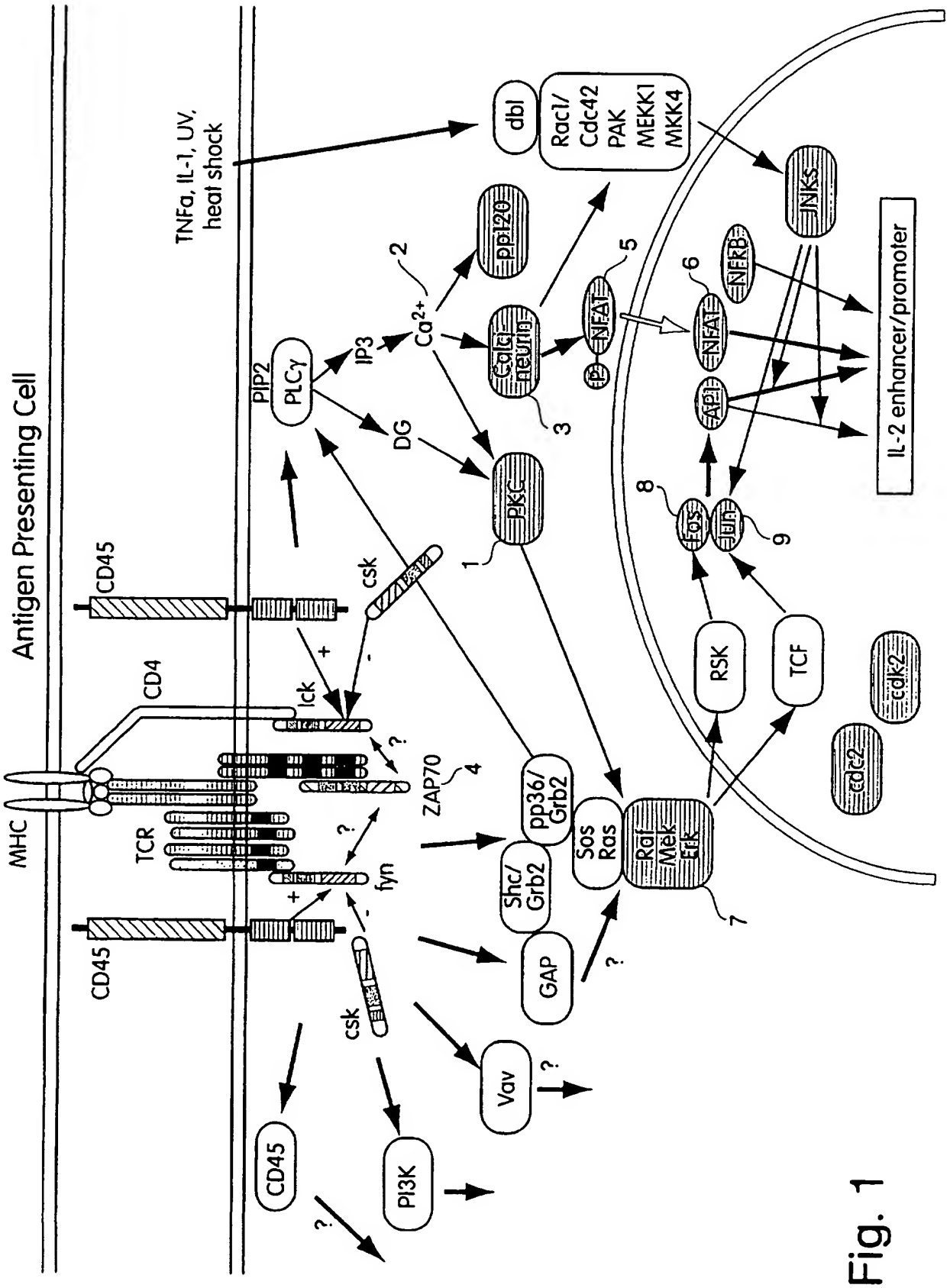

Antigen Presenting Cell
MHC
TCR
CD4
CD45
CD45
CD45
TNFa, IL-1, UV, heat shock
dbl
Rac1/ Cdc42 PAK MEKK1 MKK4
JNKs
ppi20
2
Ca2+
PIP2
PLCγ
IP3
DG
Calcineurin
3
NFAT
5
6
NFKB
NFAT
AP1
IL-2 enhancer/promoter
PKC
1
8
fos
jun
9
csk
+
-
lck
?
ZAP70
4
?
fyn
+
-
csk
pp36/ Grb2
Shc/ Grb2
GAP
Sos Ras
Raf Mek Erk
7
RSK
TCF
cdk2
cdc2
Vav
?
PI3K
?
CD45
?

Fig. 1

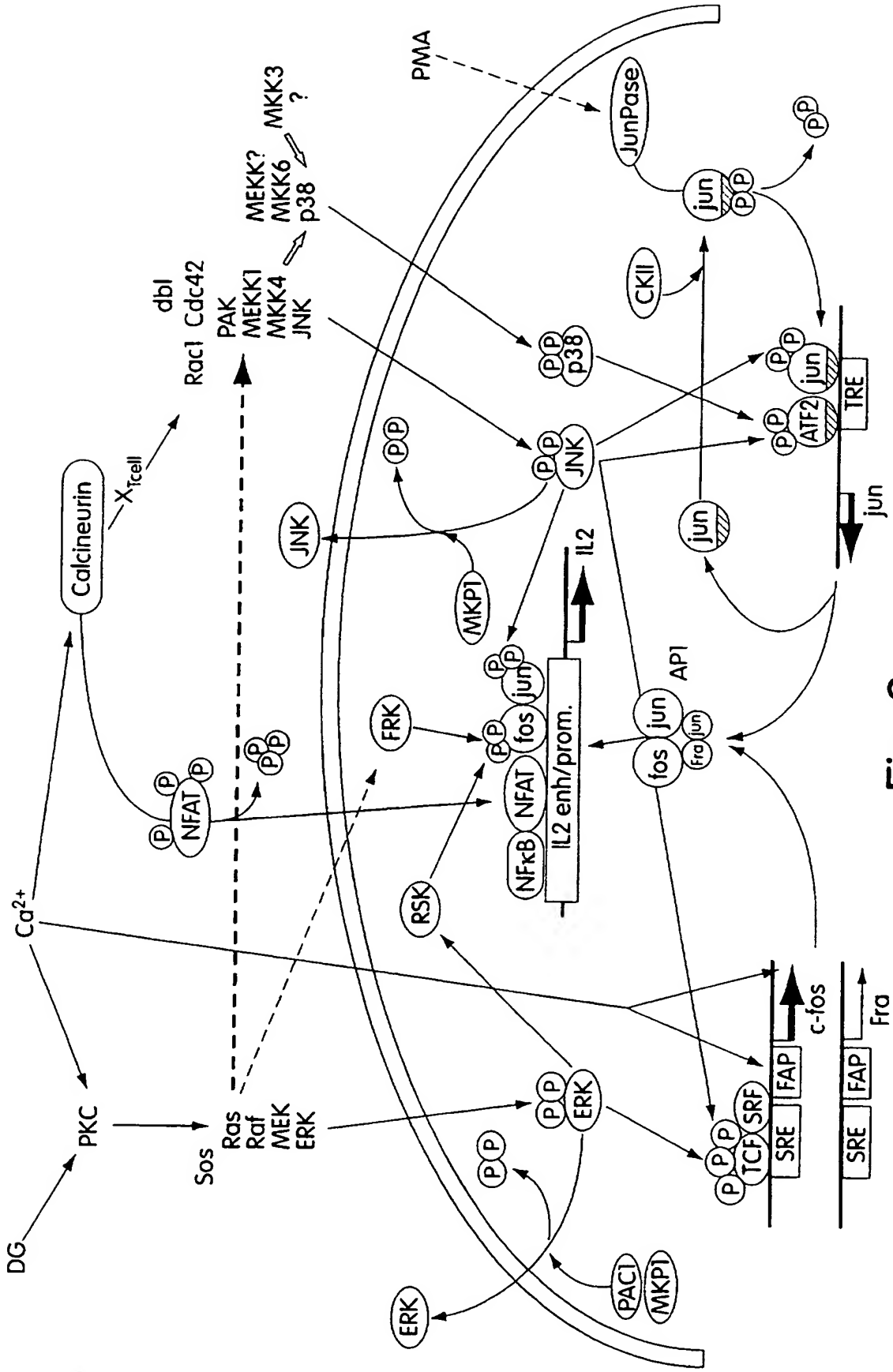

PMA
JunPase
MEKK?
MKK6
p38
MKK3
?
dbl
Rac1 Cdc42
PAK
MEKK1
MKK4
JNK
CKII
p38
JNK
jun
ATF2
TRE
Calcineurin
X_{Tcell}
MKP1
IL2
AP1
FRK
fos
Fra
NFAT
NFκB
IL2 enh/prom.
RSK
Ca^{2+}
PKC
DG
Sos
Ras
Raf
MEK
ERK
TCF
SRF
SRE
FAP
c-fos
Fra
PAC1
MKP1

Fig. 2

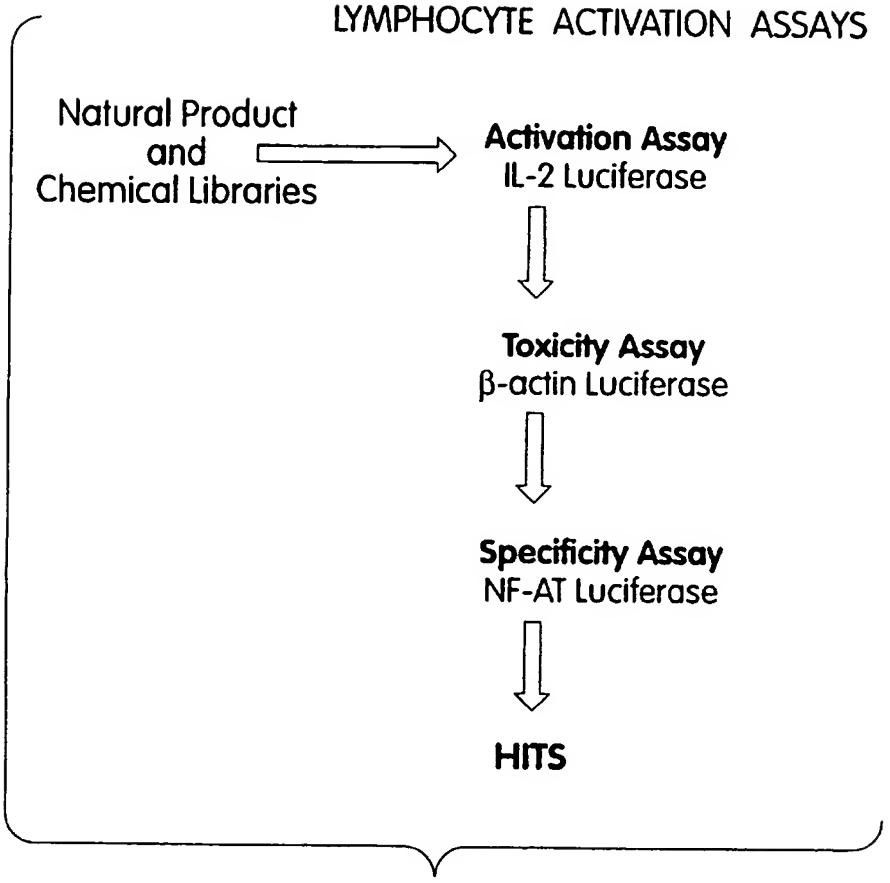

LYMPHOCYTE ACTIVATION ASSAYS
Natural Product
and
Chemical Libraries
Activation Assay
IL-2 Luciferase
Toxicity Assay
β-actin Luciferase
Specificity Assay
NF-AT Luciferase
HITS

Fig. 3

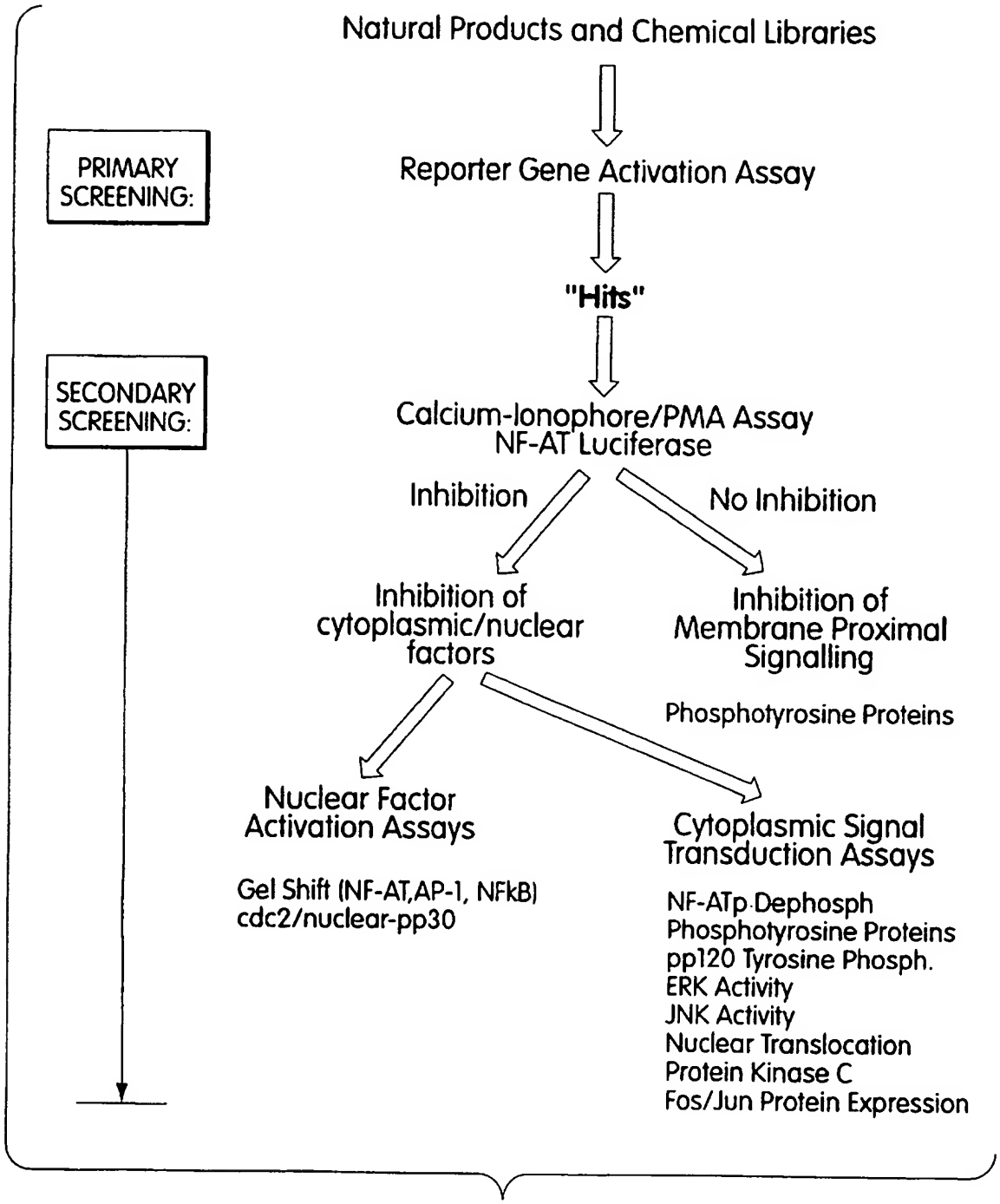

Natural Products and Chemical Libraries
PRIMARY SCREENING:
Reporter Gene Activation Assay
"Hits"
SECONDARY SCREENING:
Calcium-Ionophore/PMA Assay
NF-AT Luciferase
Inhibition
No Inhibition
Inhibition of cytoplasmic/nuclear factors
Inhibition of Membrane Proximal Signalling
Phosphotyrosine Proteins
Nuclear Factor Activation Assays
Gel Shift (NF-AT,AP-1, NFkB)
cdc2/nuclear-pp30
Cytoplasmic Signal Transduction Assays
NF-ATp·Dephosph
Phosphotyrosine Proteins
pp120 Tyrosine Phosph.
ERK Activity
JNK Activity
Nuclear Translocation
Protein Kinase C
Fos/Jun Protein Expression

Fig. 4

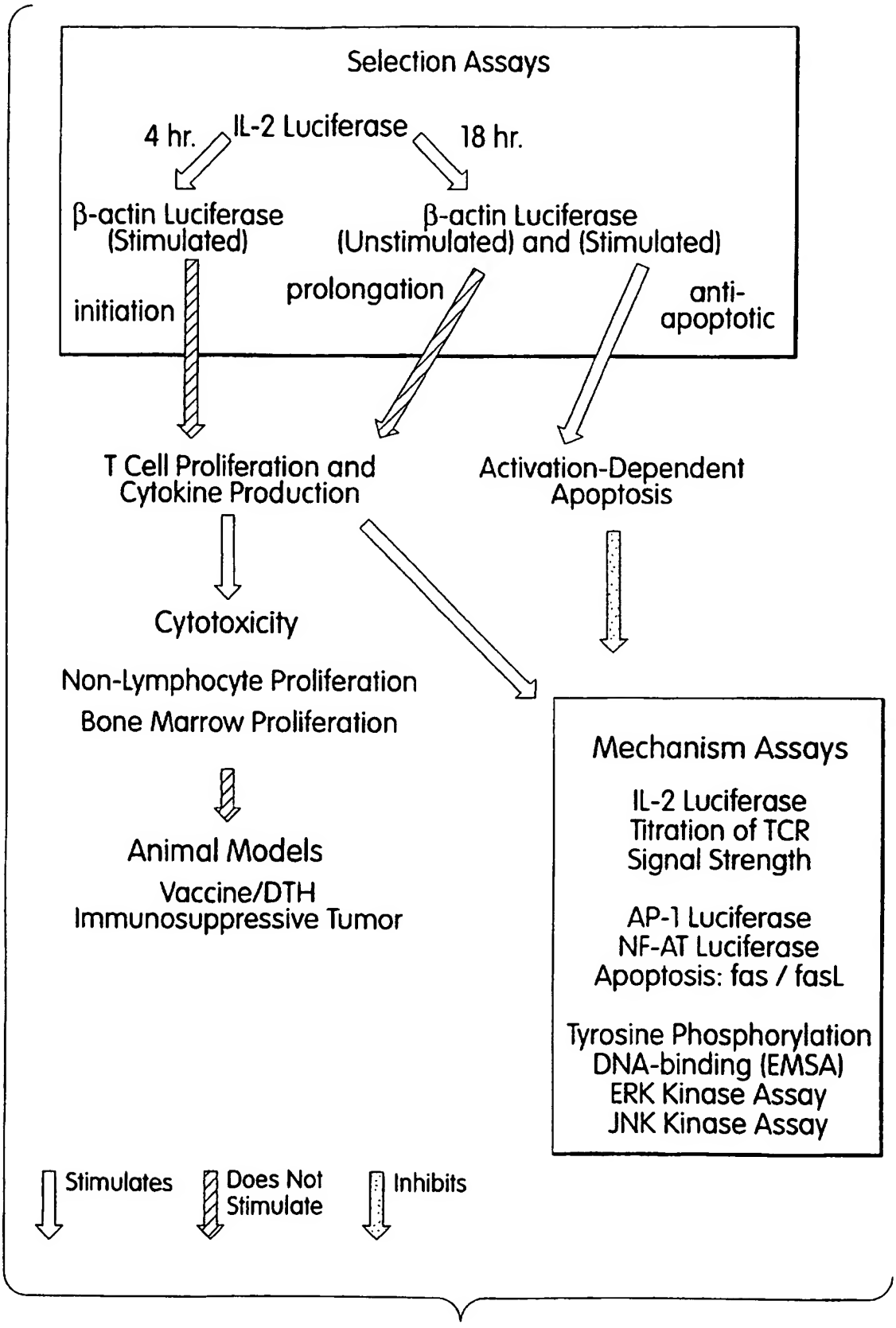
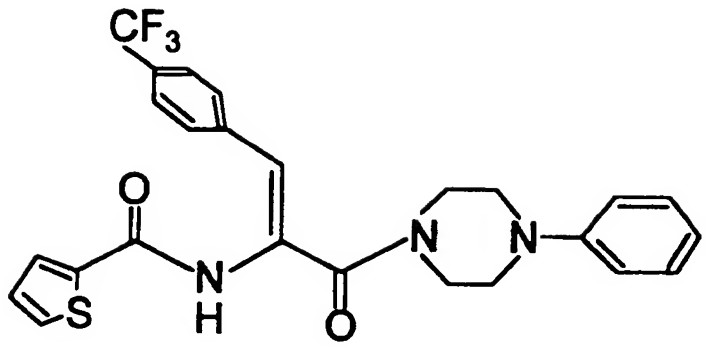
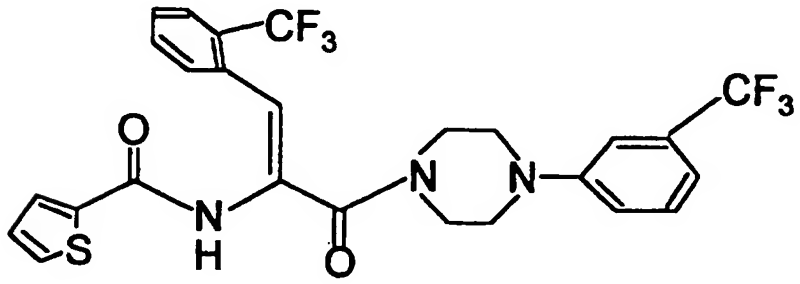

Selection Assays
4 hr.
IL-2 Luciferase
18 hr.
β-actin Luciferase (Stimulated)
β-actin Luciferase (Unstimulated) and (Stimulated)
initiation
prolongation
anti-apoptotic
T Cell Proliferation and Cytokine Production
Activation-Dependent Apoptosis
Cytotoxicity
Non-Lymphocyte Proliferation
Bone Marrow Proliferation
Animal Models
Vaccine/DTH
Immunosuppressive Tumor
Mechanism Assays
IL-2 Luciferase
Titration of TCR Signal Strength
AP-1 Luciferase
NF-AT Luciferase
Apoptosis: fas / fasL
Tyrosine Phosphorylation
DNA-binding (EMSA)
ERK Kinase Assay
JNK Kinase Assay
Stimulates
Does Not Stimulate
Inhibits

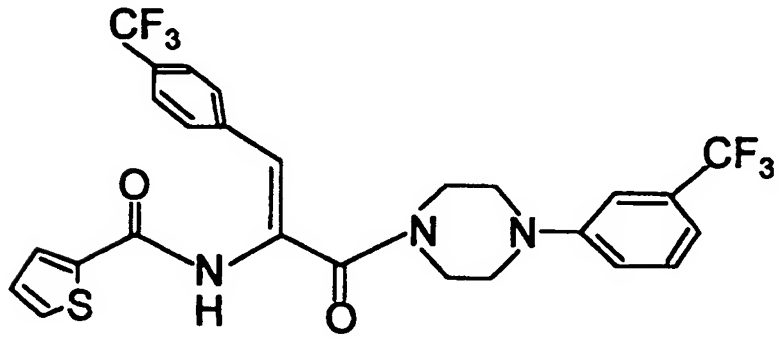
Fig. 5



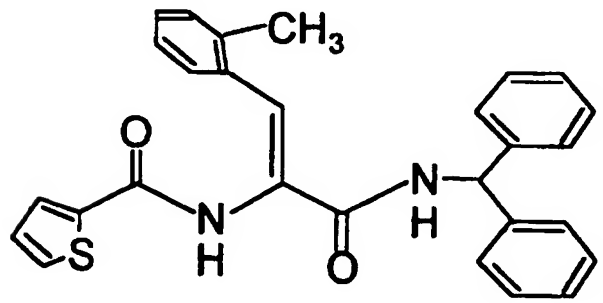
[AT-1/AQ 32778]



[AT-2/AQ 32705]

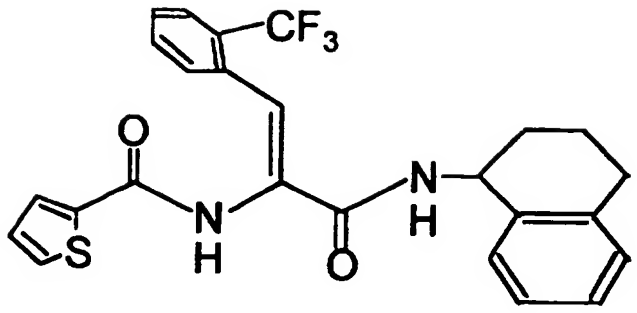


[AT-3/AQ 32785]

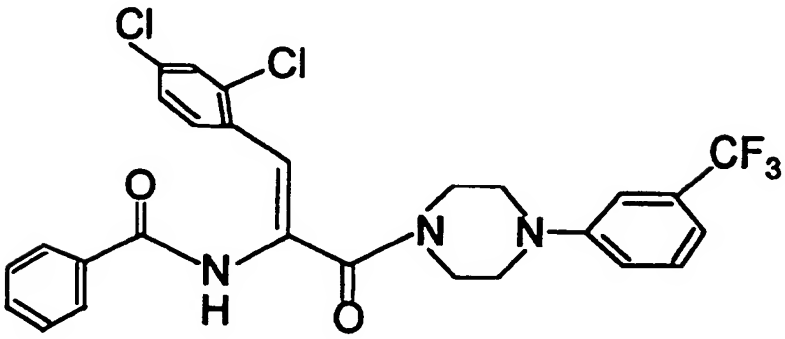


[AT-4/AQ 32809]

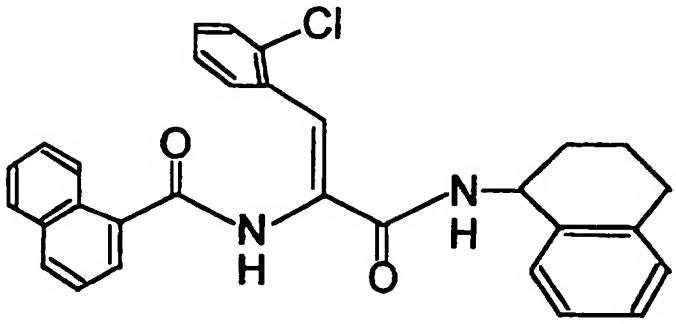
Fig. 6A



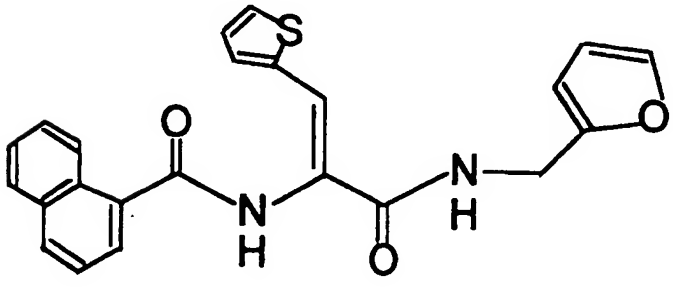
[AT-5/AQ 32687]



[AT-6/AQ 29268]



[AT-7/AQ 25958]



[AT-8/AQ 588]

Fig. 6B

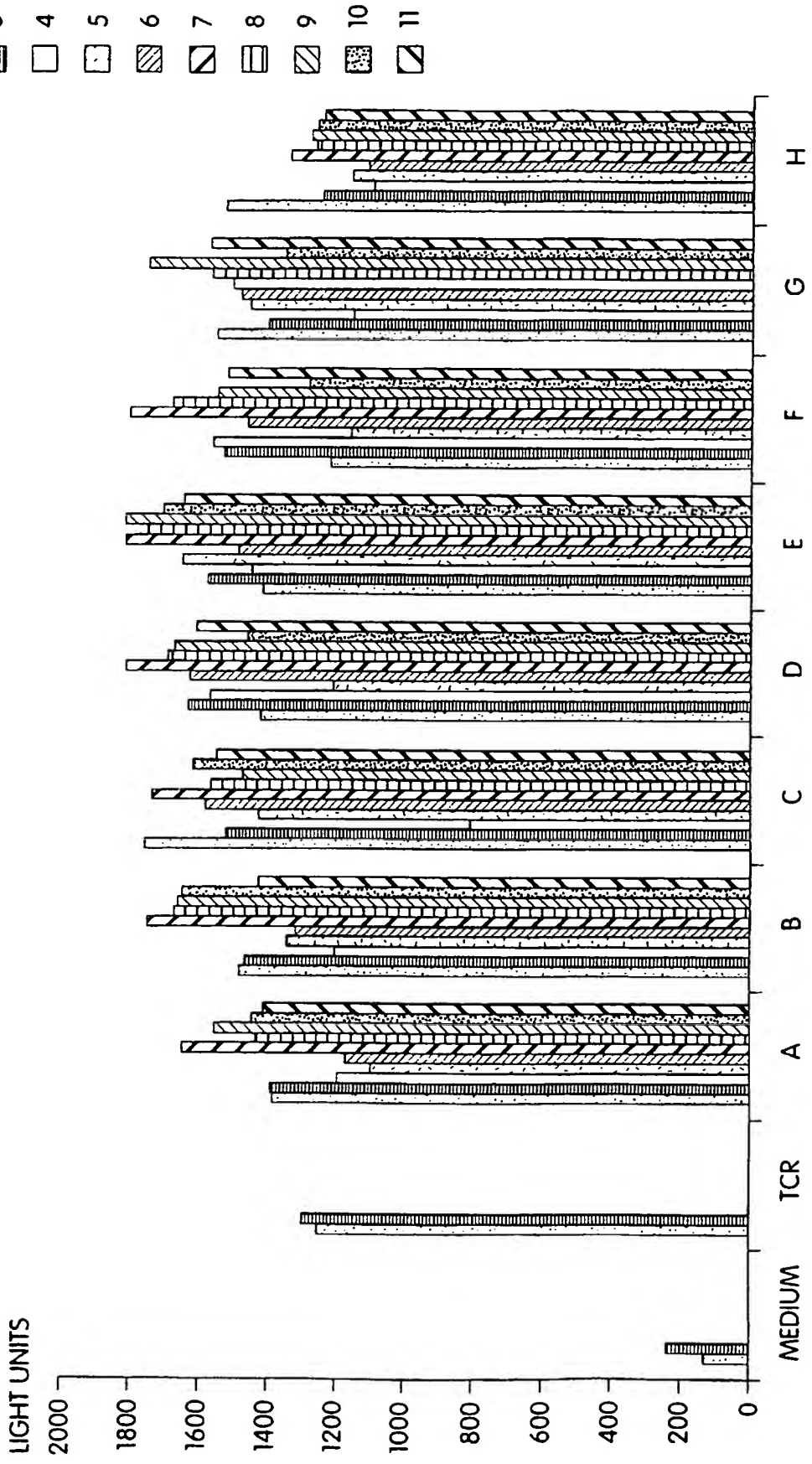

2
3
4
5
6
7
8
9
10
11
LIGHT UNITS
2000
1800
1600
1400
1200
1000
800
600
400
200
0
MEDIUM
TCR
A
B
C
D
E
F
G
H

Fig. 7

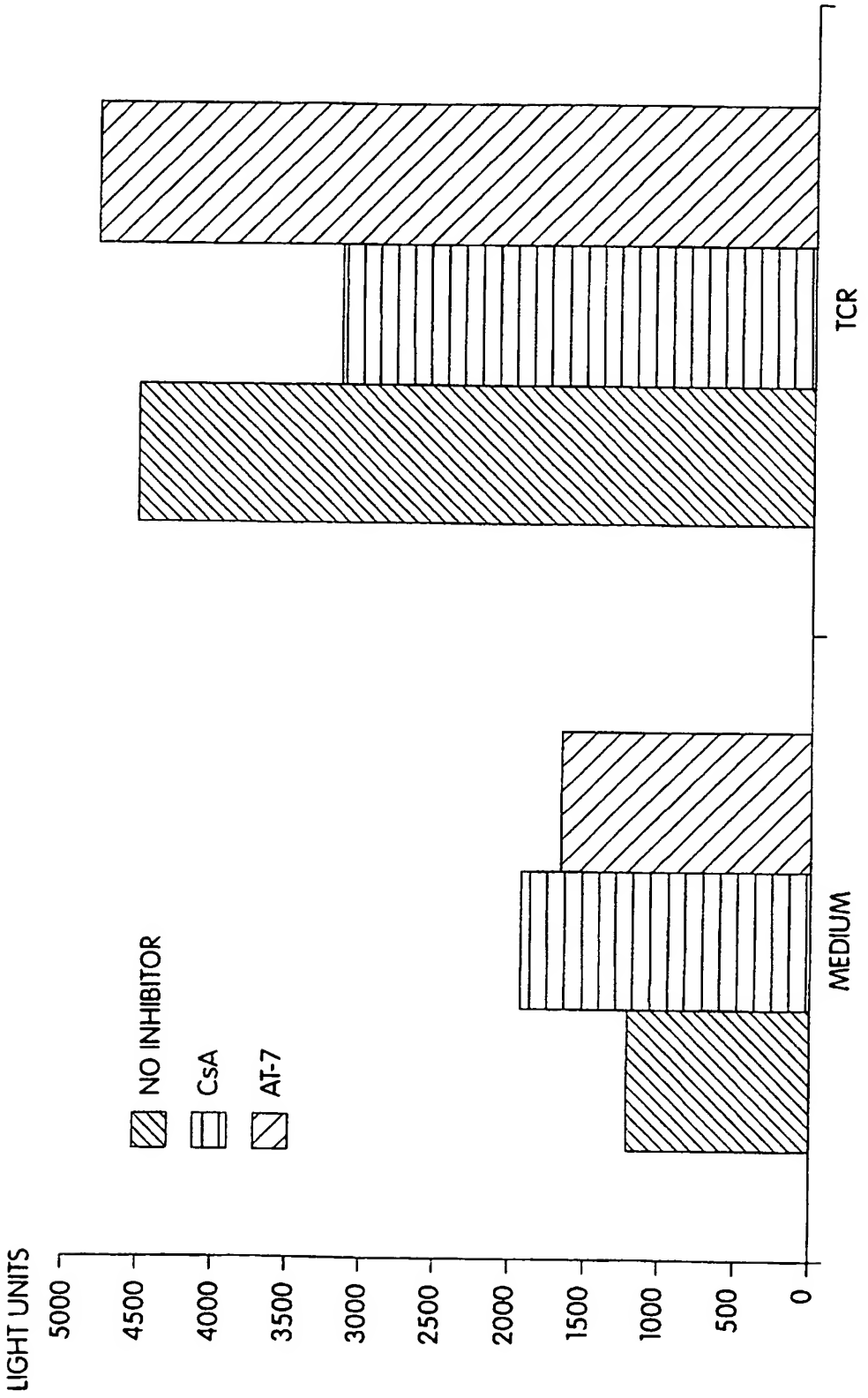

LIGHT UNITS
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
NO INHIBITOR
CsA
AT-7
MEDIUM
TCR

Fig. 8

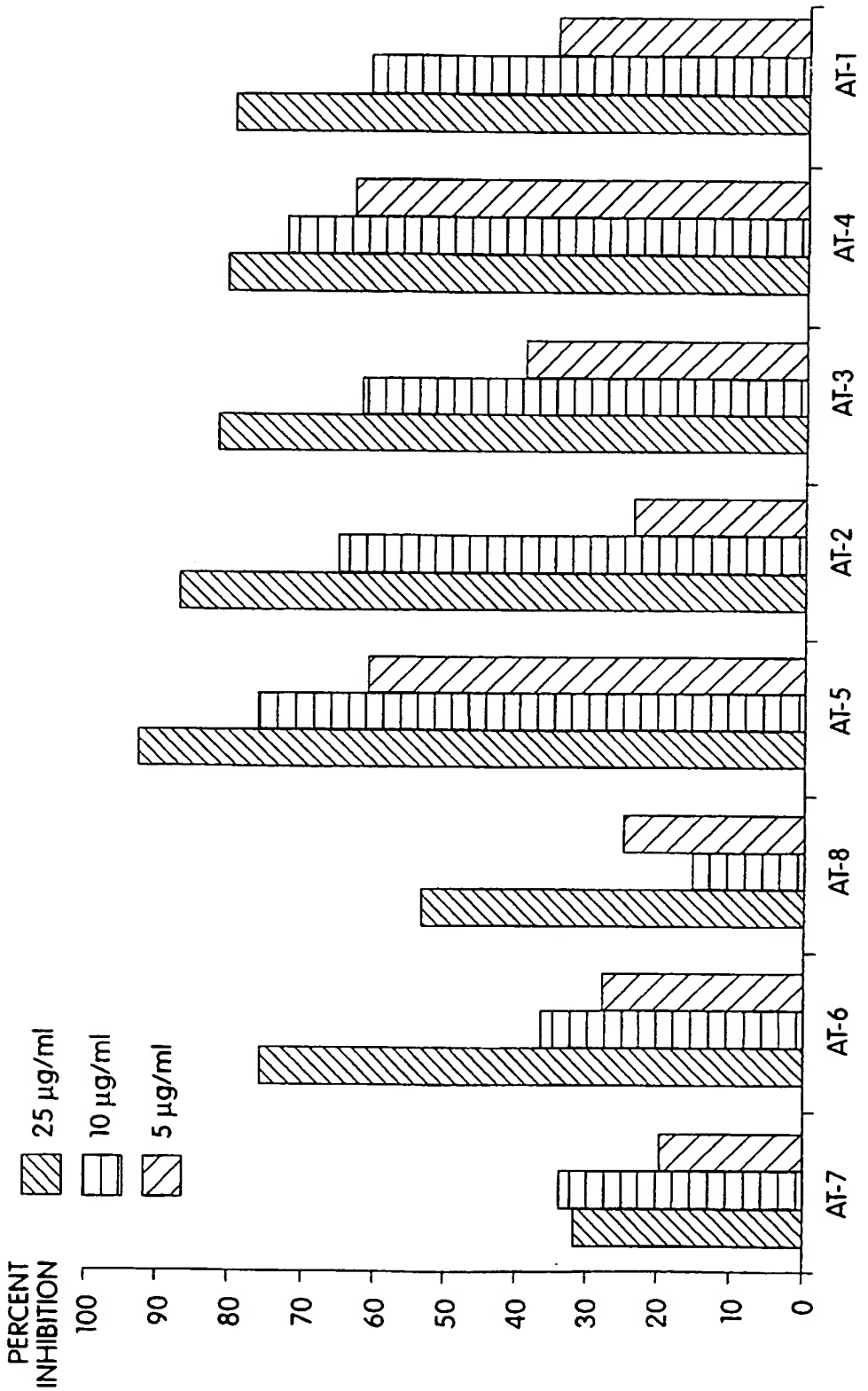

PERCENT INHIBITION
100
90
80
70
60
50
40
30
20
10
0
25 µg/ml
10 µg/ml
5 µg/ml
AT-7
AT-6
AT-8
AT-5
AT-2
AT-3
AT-4
AT-1

Fig. 9

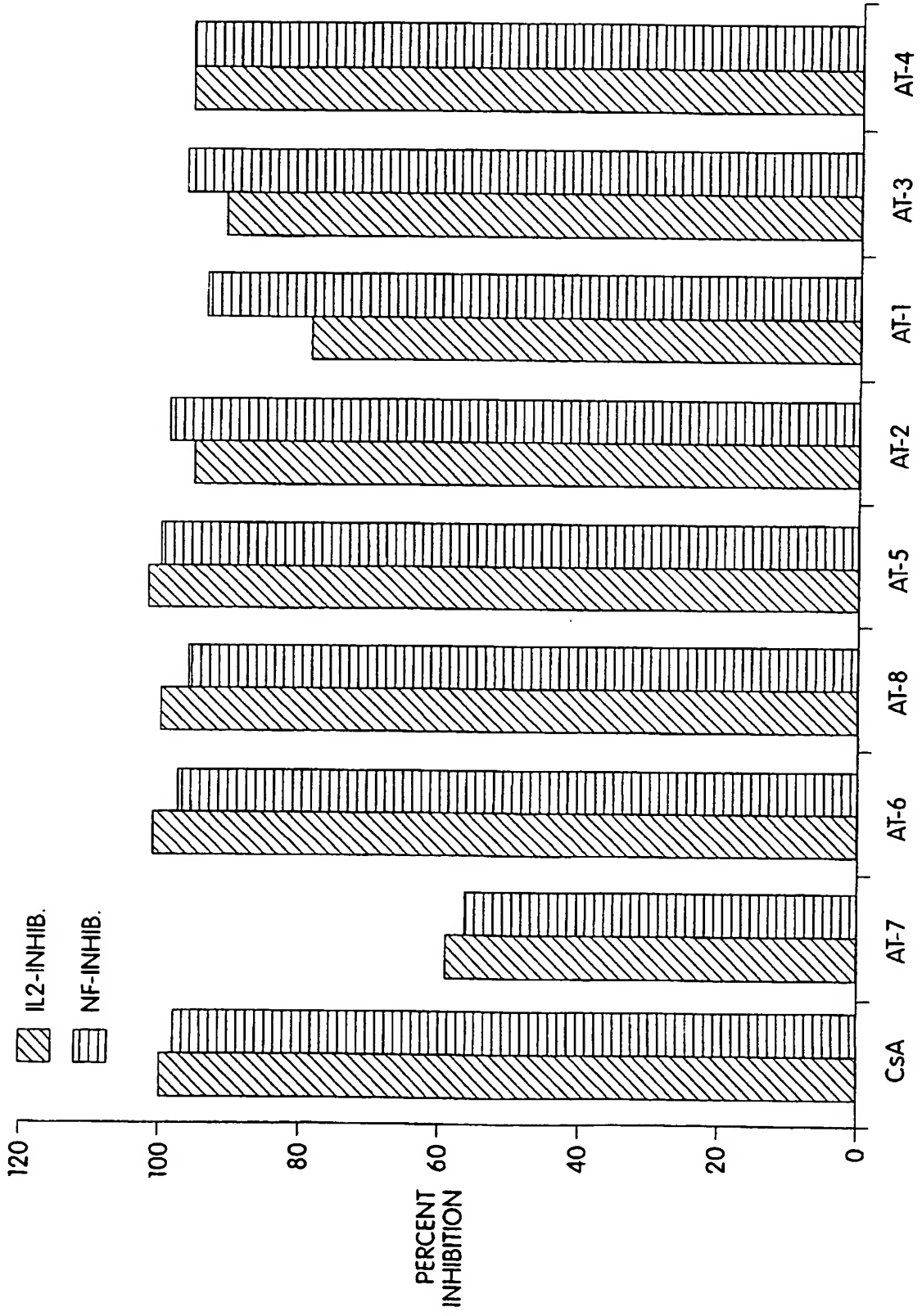

IL2-INHIB.
NF-INHIB.
PERCENT INHIBITION
120
100
80
60
40
20
0
CsA
AT-7
AT-6
AT-8
AT-5
AT-2
AT-1
AT-3
AT-4

Fig. 10

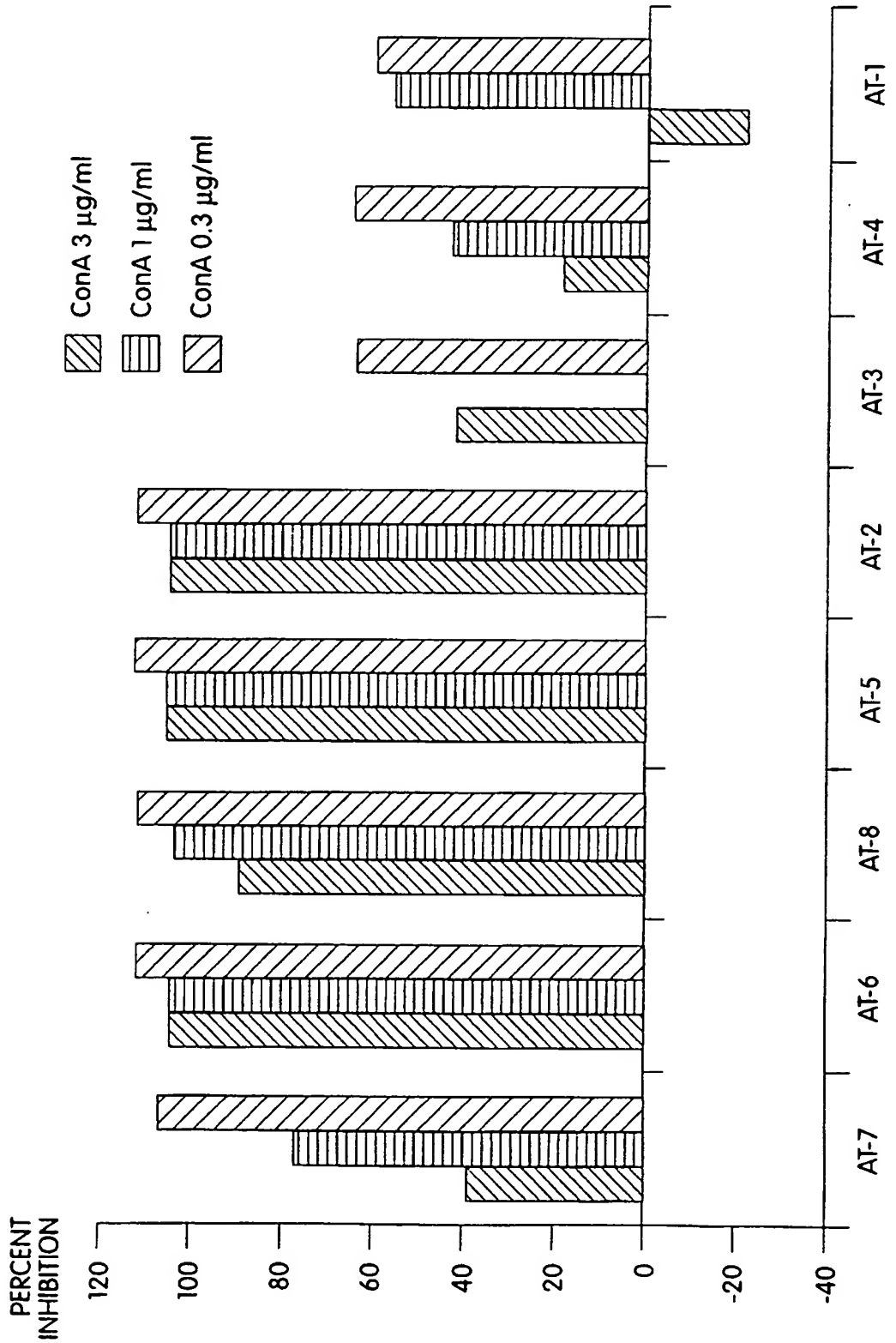

ConA 3 µg/ml
ConA 1 µg/ml
ConA 0.3 µg/ml
PERCENT INHIBITION
120
100
80
60
40
20
0
-20
-40
AT-1
AT-4
AT-3
AT-2
AT-5
AT-8
AT-6
AT-7

Fig. 11

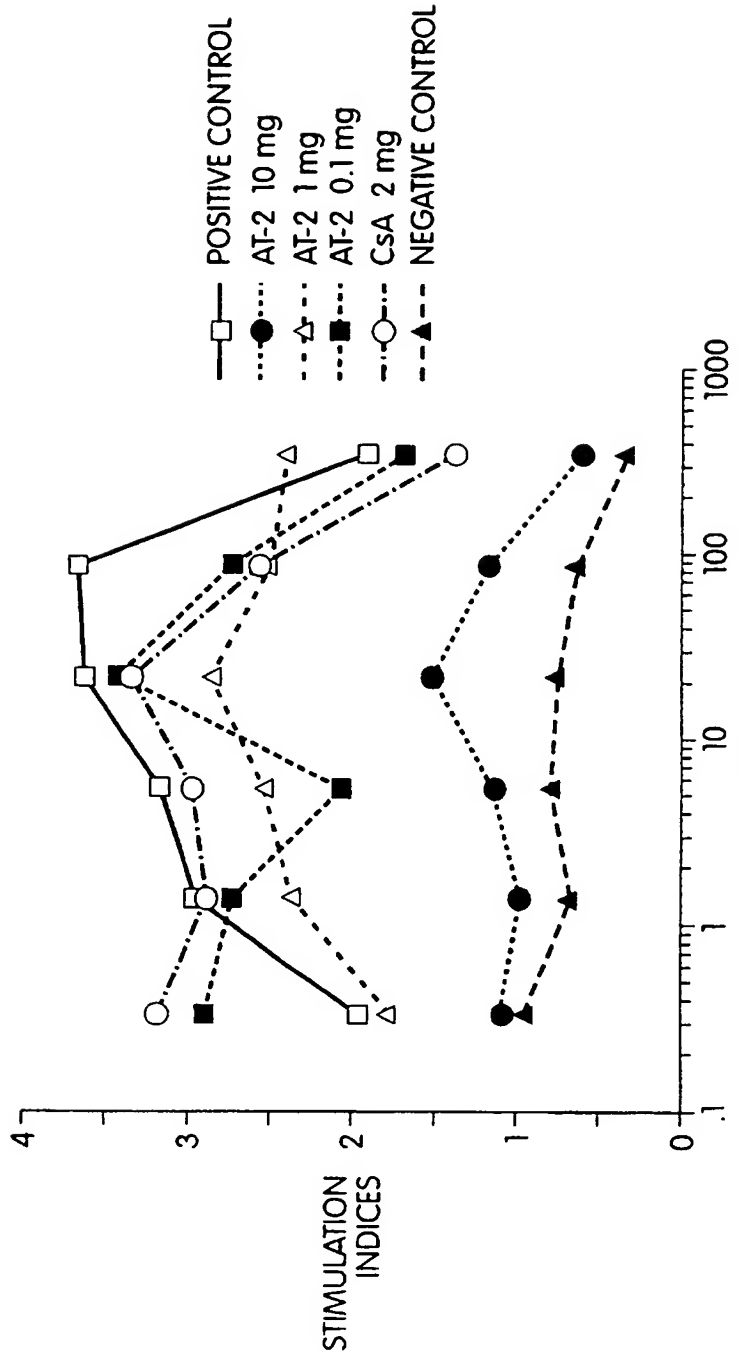

POSITIVE CONTROL
AT-2 10 mg
AT-2 1 mg
AT-2 0.1 mg
CsA 2 mg
NEGATIVE CONTROL
STIMULATION INDICES
SWM (μM)
0
1
2
3
4
.1
1
10
100
1000

Fig. 12

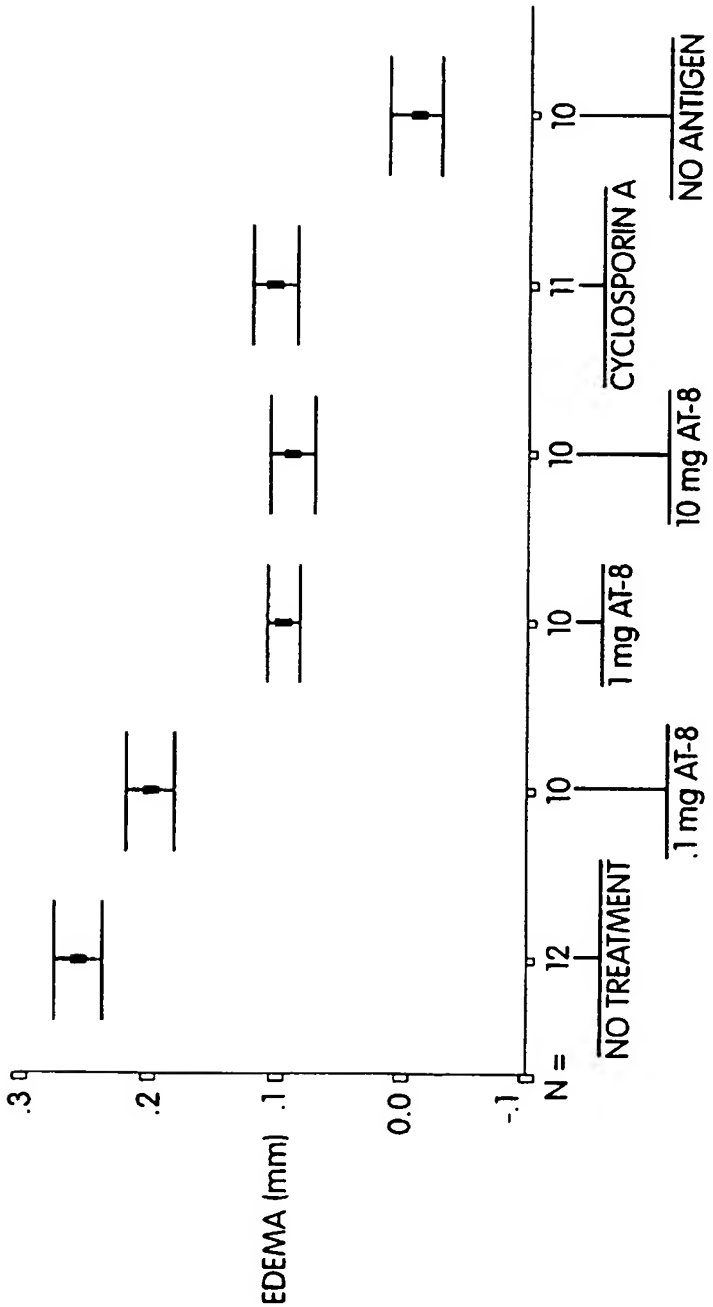

EDEMA (mm)
.3
.2
.1
0.0
-.1
N =
12
10
10
10
10
11
10
NO TREATMENT
.1 mg AT-8
1 mg AT-8
10 mg AT-8
CYCLOSPORIN A
NO ANTIGEN

Fig. 13

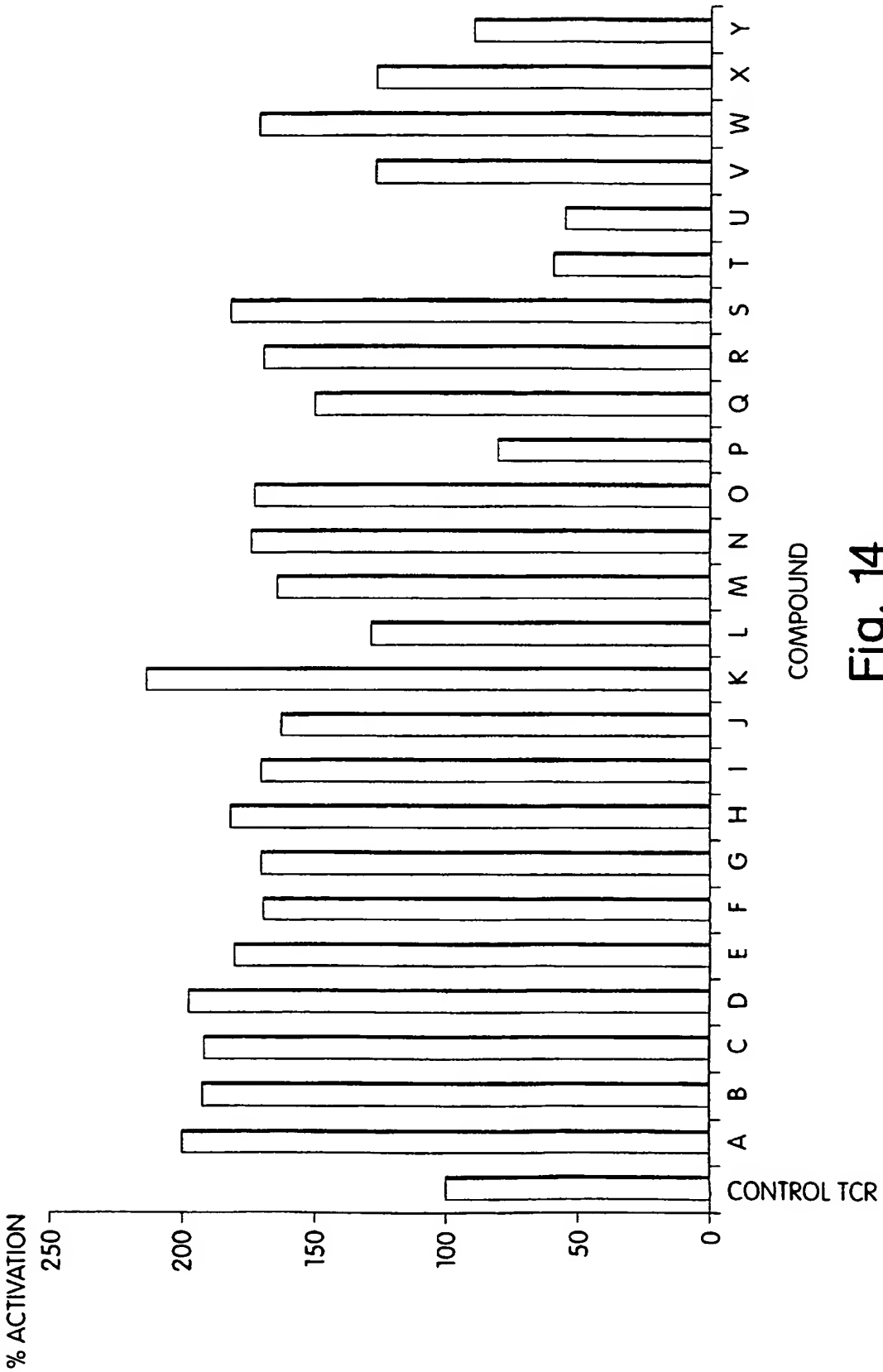

% ACTIVATION
250
200
150
100
50
0
CONTROL TCR
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
Y
COMPOUND

Fig. 14

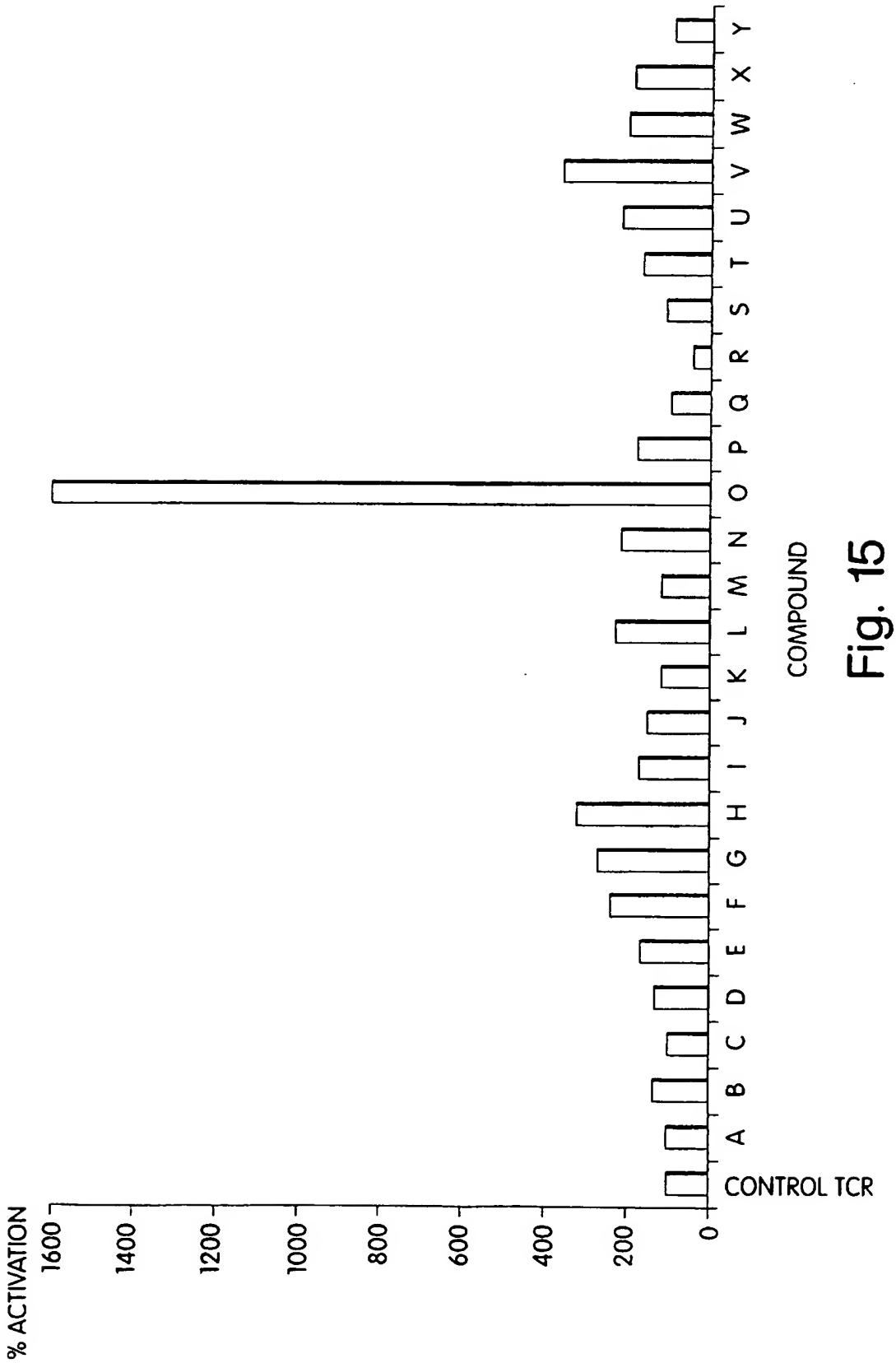

% ACTIVATION
1600
1400
1200
1000
800
600
400
200
0
CONTROL TCR
A B C D E F G H I J K L M N O P Q R S T U V W X Y
COMPOUND

Fig. 15

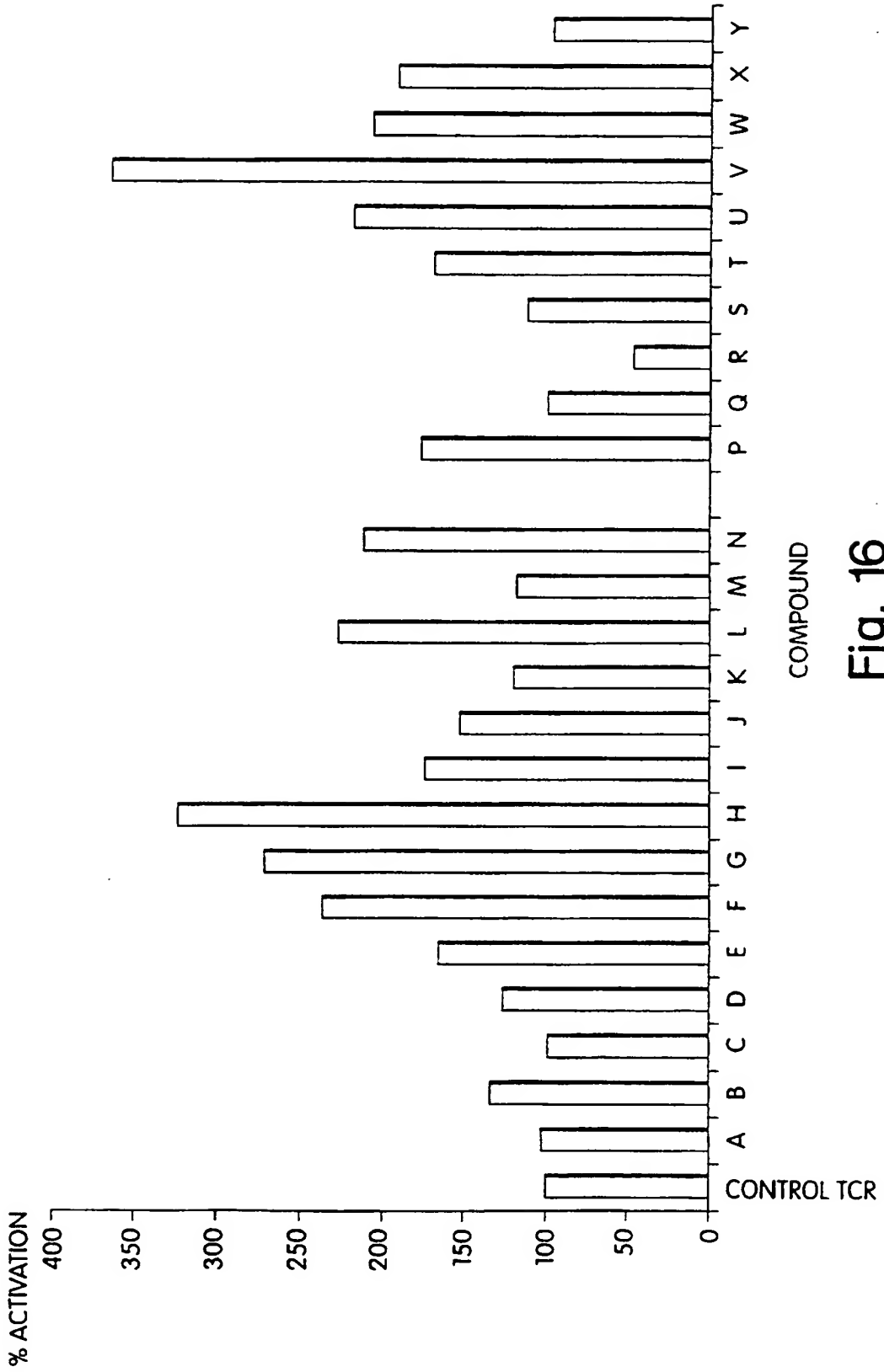

% ACTIVATION
400
350
300
250
200
150
100
50
0
CONTROL TCR
A
B
C
D
E
F
G
H
I
J
K
L
M
N
P
Q
R
S
T
U
V
W
X
Y
COMPOUND

Fig. 16

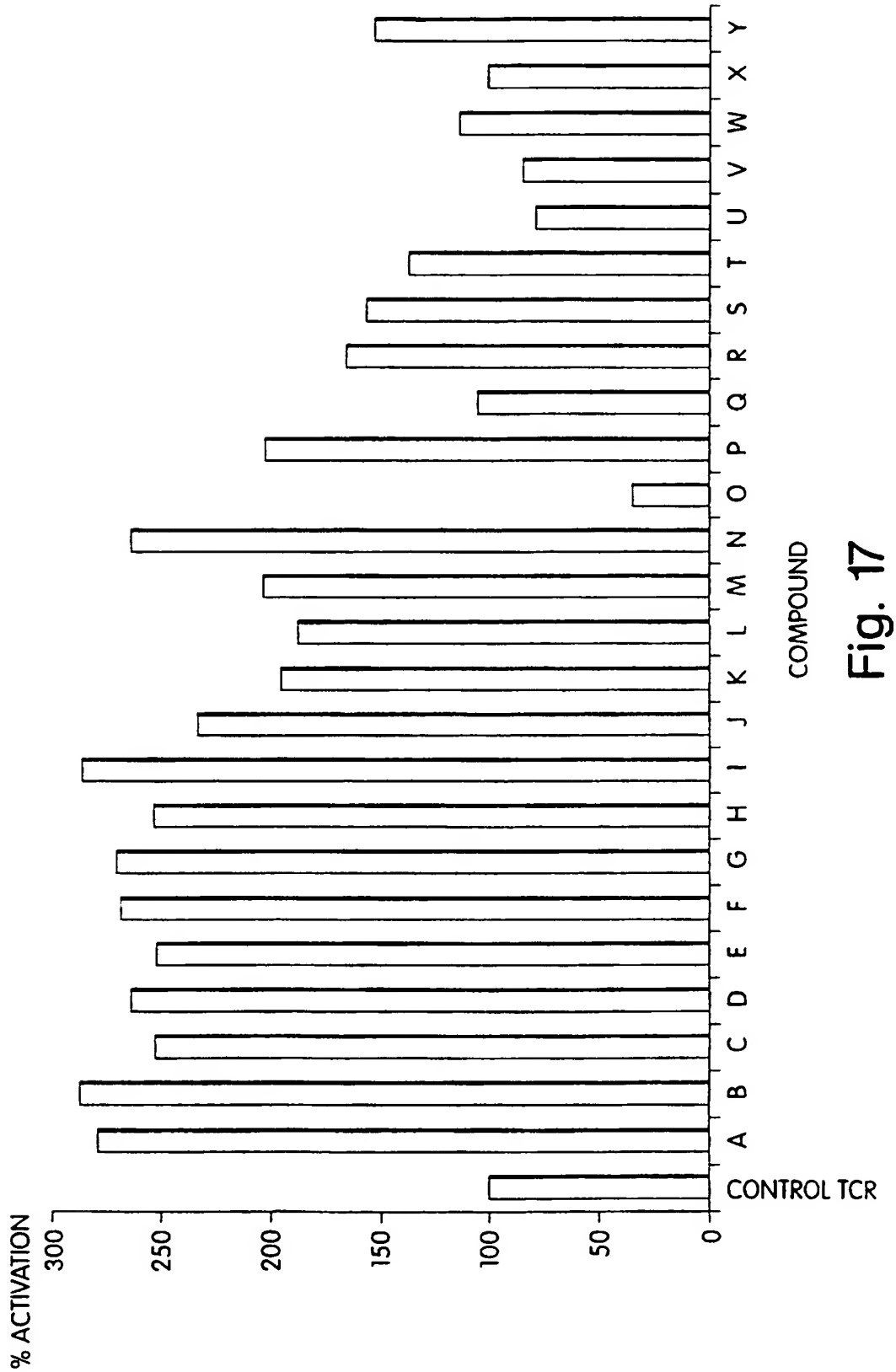

% ACTIVATION
300
250
200
150
100
50
0
CONTROL TCR
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
Y
COMPOUND

Fig. 17

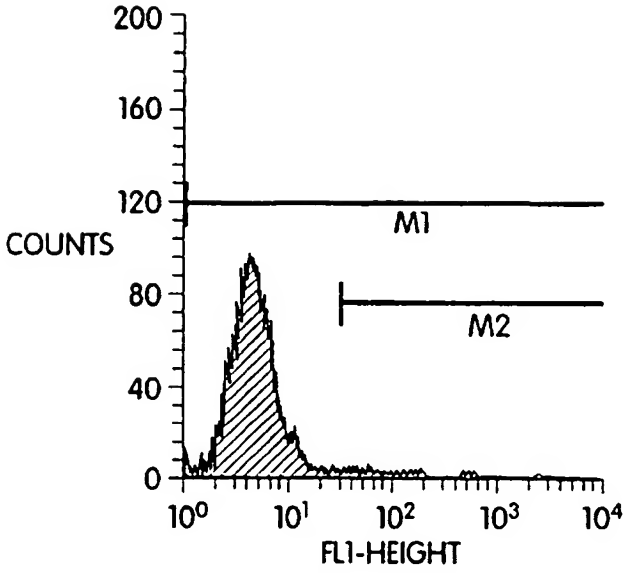


Fig. 18A

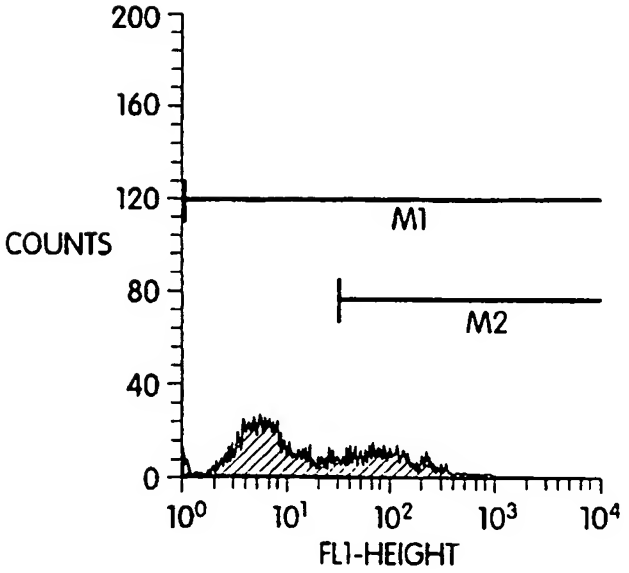


Fig. 18B

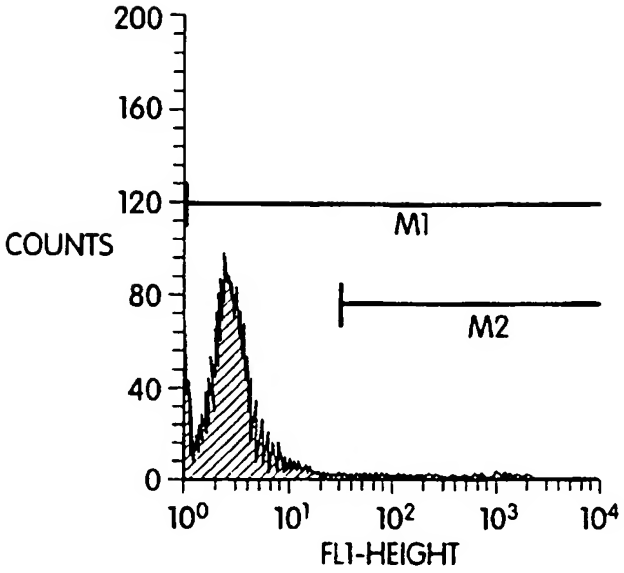


Fig. 18C

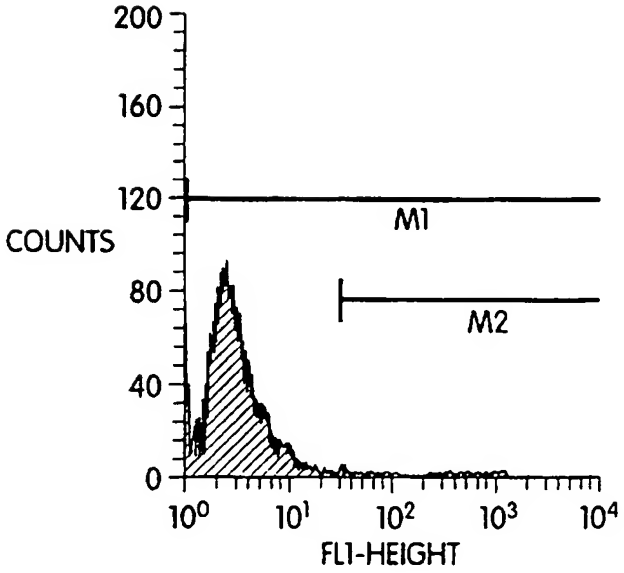


Fig. 18D

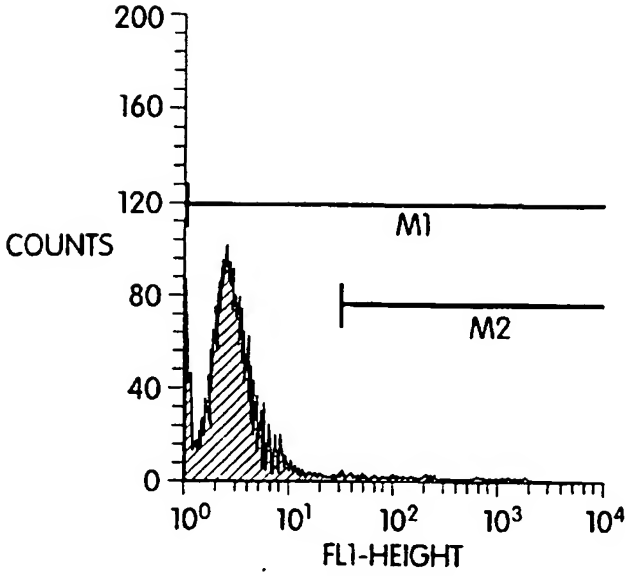


Fig. 18E

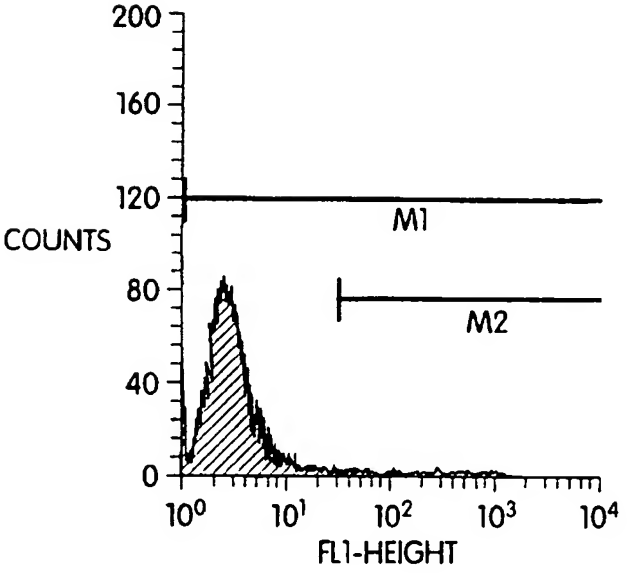


Fig. 18F

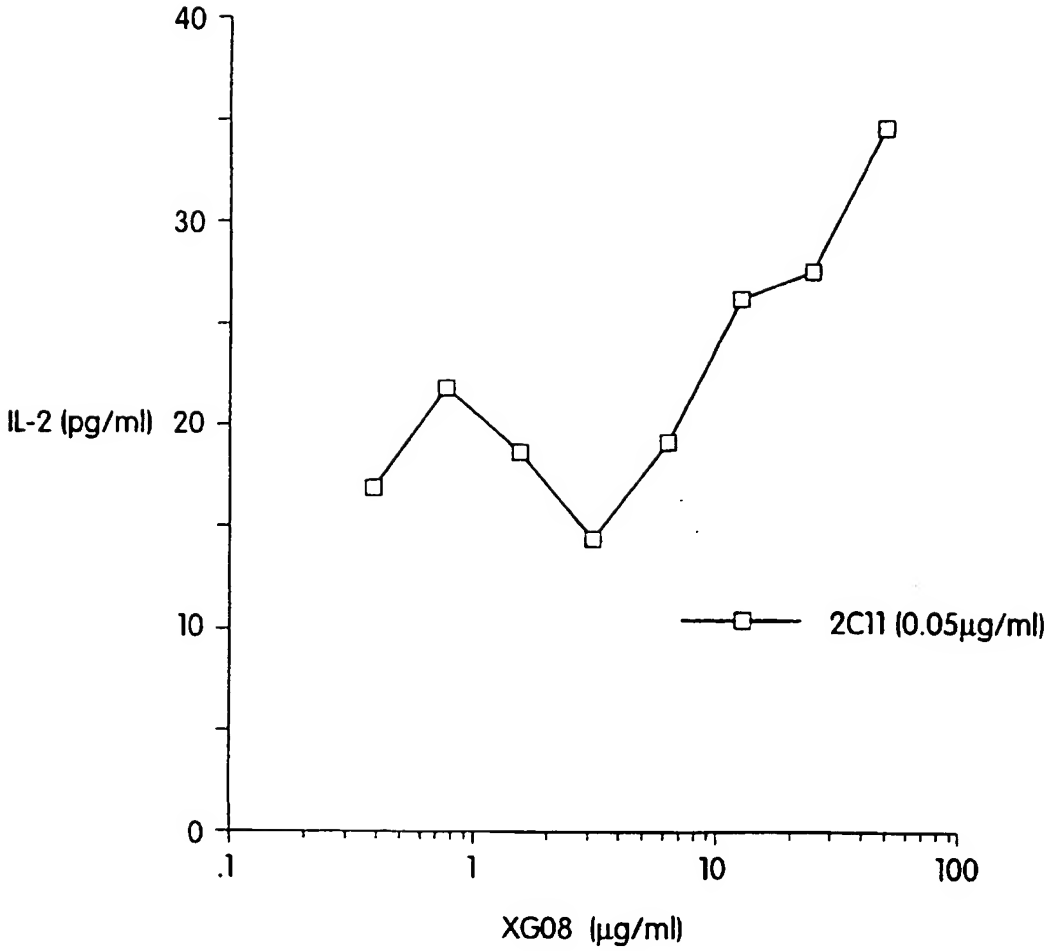
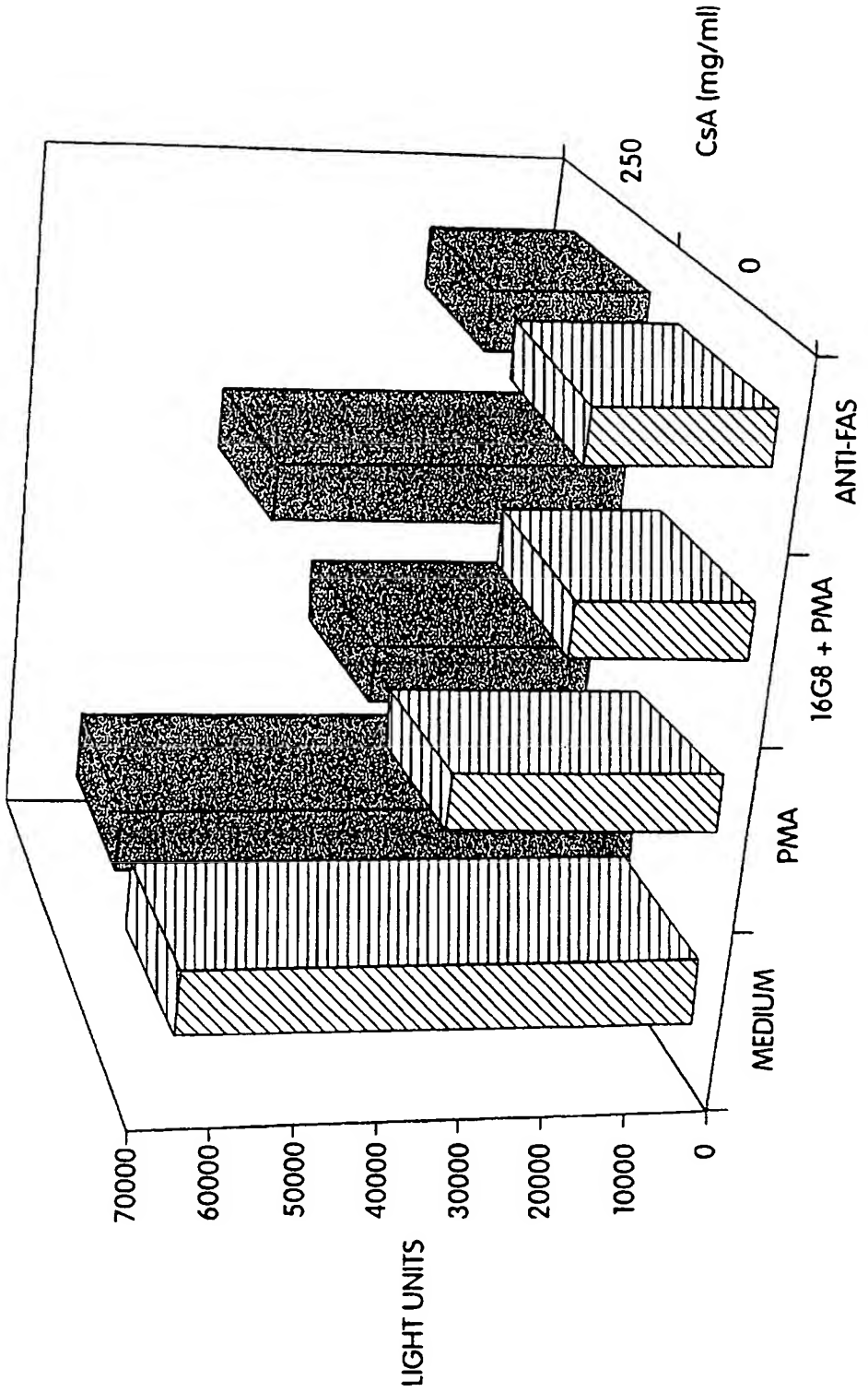


Fig. 19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ANTI-FAS
16G8 + PMA
PMA
MEDIUM
LIGHT UNITS
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60000
50000
40000
30000
20000
10000
0

Fig. 20

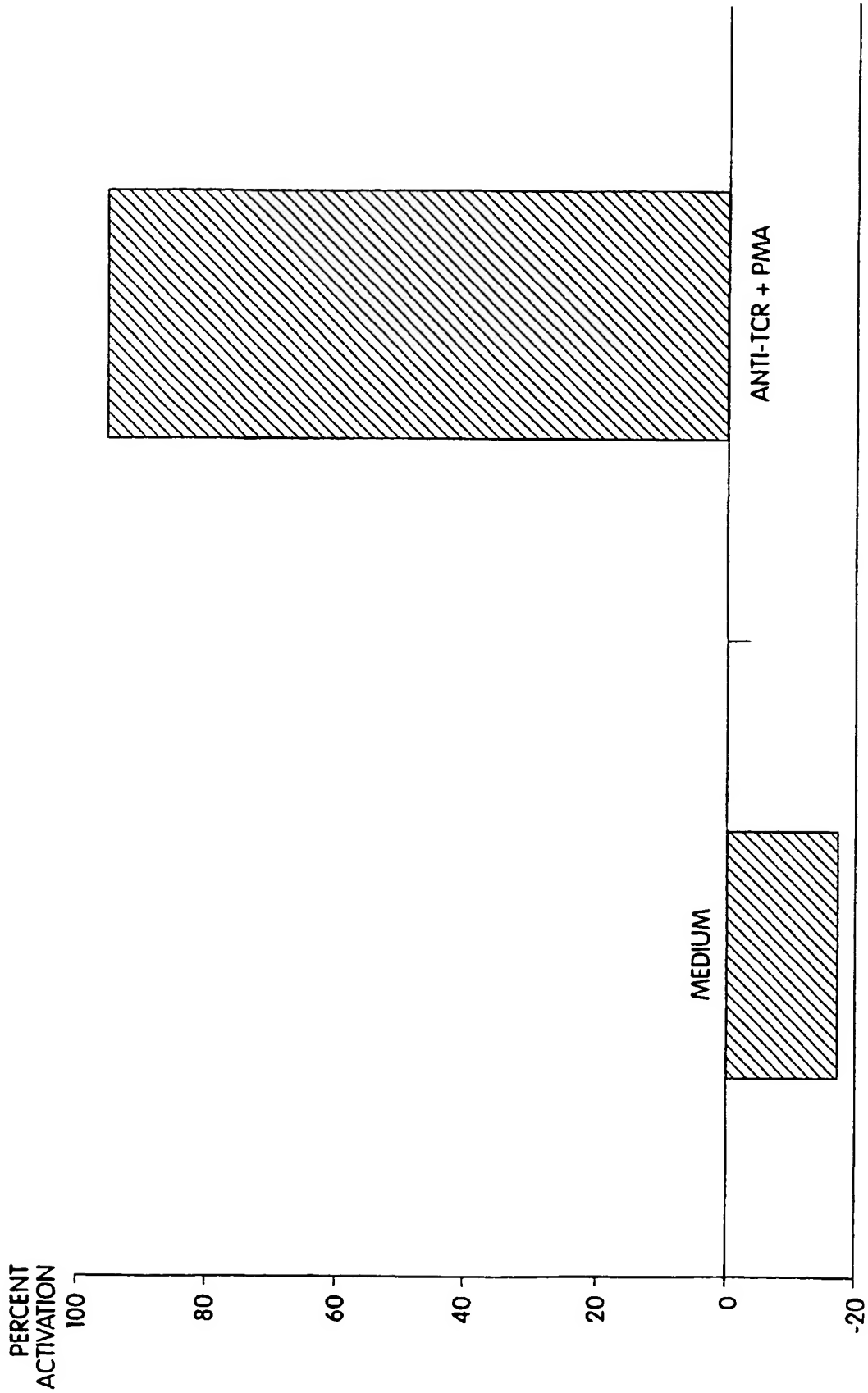

Fig. 21